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C^{14}-Glucose Incorporation into Charoninsulfuric Acids by Slices of Mucous Gland of Charonia Lampas

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(Received for publication, February 10, 1960)

Mucous gland of *Charonia lampas** contains a glyan polysulfate, which consists of only sulfate and glucose (*1*, *2*). This sulfated polysaccharide, named charoninsulfuric acid, can be separated into two fractions. One** contains much more ethereal sulfate sulfur (10–15 per cent) and its polysaccharide part is mainly composed of cellulose-like structure having $\beta_{1,4}$-glucosidic linkages. The other** contains less sulfur (1–3 per cent) and its polysaccharide part is mainly composed of amylose-like structure having $\alpha_{1,4}$-glucosidic linkages (*3*). It was also demonstrated that a parallelism existed between content of sulfur and that of cellulose-like structure in the polysaccharide part (*3*).

The characteristic feature of charoninsulfuric acids may raise several interesting problems on the biosynthesis of charoninsulfuric acids such as the relation between biological sulfation and elongation of polysaccharide chain, transformation from amylose-like structure to cellulose-like structure, or biosynthesis of cellulose-like structure in animal kingdom. Many possible pathways of the biosynthesis of charoninsulfuric acids may be proposed. Especially, if a molecule of charoninsulfuric acids contains both amylose-like structure and cellulose-like structure, transfer reaction from amylose-like structure to cellulose-like structure may be involved in the biosynthetic pathway of S-rich charoninsulfuric acid. On the other hand, if a molecule of charoninsulfuric acids contains only either of two structures of polysaccharide, the biosynthesis of two types of charoninsulfuric acids seems to be independent on each other.

Biosynthesis of charoninsulfuric acids was investigated in our laboratory from these considerations. The incorporation of S^{35}-sulfate into charoninsulfuric acids was demonstrated *in vivo* and *in vitro* (*4*). The transsulfation from *p*-nitrophenylsulfate to charoninsulfuric acid was observed with cell-free extracts and partially purified preparation (*5*). Following these investigations on the sulfation of charoninsulfuric acid, the study on the biosynthesis of polysaccharide part has been planed. It is the purpose of this communication to present that slices of mucous gland of *Charonia lampas* incorporate C^{14}-glucose into charoninsulfuric acid.

EXPERIMENTAL

C^{14}-Glucose—C^{14}-Glucose was prepared with acid-hydrolysis of C^{14}-starch, which was biosynthesized from C^{14}-carbon dioxide (*6*), or it was prepared from alcohol extracts of green leaves, which was exposed to light in the presence of C^{14}-carbon dioxide (*7*). These preparations of C^{14}-glucose were purified chromatographically.

Determination of Sulfur and Nitrogen—Sulfur contents of charoninsulfuric acids were determined as sulfate sulfur according to the method of Egami and Takahashi after acid-hydrolysis (*8*). Nitrogen contents of charoninsulfuric acids were determined with Kjeldahl method.

Paperchromatography and Radiography—Two solvent systems were used for chromatography of sugar: A; butanol, etanol, water, ammonia water (28%) (40, 12, 20, 1), B; butanol, acetic acid, water (15, 4.5, 10). The radio-activity on filter paper developed by means of ascending paperchromatography, were determined at intervals of 1 cm. with Geiger-Müller counter.

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* A marine gastropped, called "*Boshubora*" in Japanese, was identified with *Charonia lampas* (Linne), but according to T. Kira it should be identified with Tritonalia sauliae (Reeve).

** These fractions are named S-rich and S-poor charoninsulfuric acid respectively.

Experiments on the C^{14}-Glucose Incorporation with Slices—Slices were prepared in a potassium chloride isotonic medium with safety razor blade from mucous gland, which was freshly cut off from *Charonia lampas.* Slices of 300～500 mg. (wet weight) were introduced into 5 ml. incubation medium in 50 ml. vessel, which contained C^{14}-glucose (about 10^5 c.p.m.) and 1 mmol. of sodium succinate in potassium chloride isotonic solution, and the vessel was shaked at 30°C. After several hours, the reaction mixture was boiled for 1 minute and then cooled. Hydrochloric acid was added into the reaction mixture to 1.7 per cent at final concentration. Charoninsulfuric acids were extracted from the acidified reaction mixture according to the usual method described below.

Preparation of Charoninsulfuric Acids—Charoninsulfuric acids were extracted from mucous gland with diluted hydrochloric acid and fractionated in two fractions (S-poor and S-rich charoninsulfuric acids) with potassium chloride precipitation and ethanol precipitation, according to the method II previously mentioned (*3*). The charoninsulfuric acids prepared were dissolved in 1.7% hydrochloric acid, precipitated with potassium chloride or ethanol repeatedly, and dialyzed. In these experiments, S-rich charoninsulfuric acid preparation contained 13.8% sulfur and 0.28% nitrogen and S-poor charoninsulfuric acid preparation contained 4.0–5.0% sulfur. Unless any separation between S-rich charoninsulfuric acid and S-poor one was necessary, the acid extract was neutralized with potassium hydroxide and both charoninsulfuric acids together were precipitated with ethanol.

Determination of Radioactivity of Charoninsulfuric Acids—Radioactivity of the charoninsulfuric acids preparation was estimated in stainless steel planchet with Geiger-Müller counter. Radioactivity counted was corrected in responce to amounts of charoninsulfuric acids.

RESULTS AND DISCUSSION

Fig. 1 indicates that by slices of mucous gland the radioactivity of C^{14}-glucose was incorporated into charoninsulfuric acids and the radioactivity of charoninsulfuric acids were reached to 100 c.p.m. after 4 hours. No incorporation was observed when slices were incubated at 0°C. Slices previously treated at 100°C for 2 minutes, completely lost this activity.

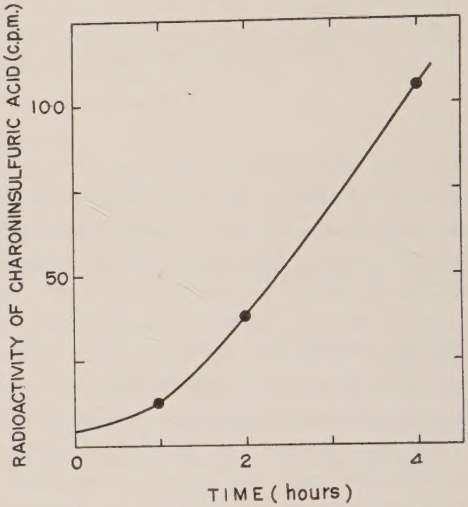

FIG. 1. C^{14}-Glucose incorporation into charoninsulfuric with slices of mucous gland.

Potassium chloride isotonic solution contained $1.34\times10^{-1}M$ KCl, $2.87\times10^{-3}M$ $CaCl_2$, $1.34\times10^{-3}M$ KH_2PO_4, $1.34\times10^{-3}M$ $MgCl_2$, $5.36\times10^{-3}M$ NaCl, and $5.22\times10^{-3}M$ Na_2HPO_4.

Other reaction condition was same as in text.

TABLE I

Comparison of Amounts of C^{14}-Glucose Incorporation into the Two Fractions; S-poor Charoninsulfuric Acid and S-rich Charoninsulfuric Acid

	Amount of C^{14}-glucose incorporated	
	S-poor Charoninsulfuric acid	S-rich Charoninsulfuric acid
Exp. 1	58 c.p.m.	46 c.p.m.
2	146	95

When C^{14}-glucose (2×10^5 c.p.m., 0.2 μmole) was added into the same system mentioned above, 650 c.p.m. of C^{14}-glucose was incorporated into charoninsulfuric acids. In Table I, the comparison of the activities incorporated into the two fractions of charoninsulfuric acids (S-poor and S-rich charoninsulfuric acids) were listed. Table I indicates that C^{14}-glucose was incorporated slightly more into S-poor fraction than S-rich fraction. The replacement of air by oxygen or nitrogen and that of potassium chloride isotonic medium by Krebs-Ringer's solution did not affected the activity of incorporation of C^{14}-glucose by slices. The incorporation of C^{14}-glucose was inhibited by 2,4-

dinitrophenol. These results are summarized in Table II.

The amounts of incorporated C^{14}-glucose were corresponded to 0.2–0.3 per cent of those added into the reaction mixture and were 0.8–2.4 mμmole in 4 hours by 1 g. slices of wet weight, if not diluted with any intermediates which were contained in slices.

TABLE II

C^{14}-Glucose Incorporation by Slices in Various Conditions

Reaction conditions		C^{14}-Radioactivity of charoninsulfuric acids
Exp. 1	Reaction medium	
	KCl	124 c.p.m.
	NaCl[1]	172
Exp. 2	Gas phase	
	air	170
	oxygen	175
	nitrogen	200
Exp. 3		
	Complete system	42
	+dinitrophenol	5

The reaction conditions were same as in text except mentioned in this table. 1) Krebs-Ringer's solution was indicated with NaCl.

As shown in Table III, the radioactivity of charoninsulfuric acids was not decreased with reprecipitation or dialysis. Determination of nitrogen contents of charoninsulfuric acids preparation indicated that amounts of protein contaminated were very small. The residue of slices, from which charoninsulfuric acids were extracted, was shown to incorporate only 10～20 c.p.m. of C^{14}-glucose (5–10 per cent of that incorporated into charoninsulfuric acids fractions). These results lead us to the conclusion that non-dialysable radioactivity of charoninsulfuric acid preparation is due to C^{14}-glucose incorporated into glucan part of charoninsulfuric acids.

TABLE III

Treatment of C^{14}-Labeled Charoninsulfuric Acids

	Radioactivity of Charoninsulfuric Acids	
	Exp. 1.	Exp. 2.
	c.p.m./mg.	c.p.m./mg.
Original	4.0	15.5
After reprecipitation[1]	3.8	—
After dialysis	3.5	15.9

1) After a solution of C^{14}-labeled charoninsulfuric acids was incubated with non-labeled glucose at 30°C for 5 hours, charoninsulfuric acids were re-precipitated.

Radioactivity of C^{14}-labeled charoninsulfuric acids was retained at original point after developing chromatographically with solvent A or B, while glucose was migrated about 10 cm. It was also indicated chromatographically that acid hydrolysis of labeled charoninsulfuric acids yielded C^{14}-glucose.

SUMMARY

C^{14}-Glucose was incorporated into charoninsulfuric acids by mucous gland of *Charonia lampas*. Amounts of the incorporated glucose were corresponded to 0.2–0.3 per cent of that added into the reaction mixture. The radioactivity of charoninsulfuric acids did not decrease by a dialysis. The glucose incorporation reaction was inhibited by 2,4-dinitrophenol. The rate of glucose incorporation into S-poor charoninsulfuric acid was slightly faster than that into S-rich charoninsulfuric acid.

The author wishes to express his gratitude to Prof. F. Egami for his useful advice. This research was supported by grants from the Scientific Research Found of the Ministry of Education and Seikagaku-kenkyusho Ltd. Some of the experiments were carried out in the Marine Biological Station of Nagoya University.

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On the Accumulation of Noradrenaline in Guinea Pig Brain Mitochondrial Particles

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(Received for publication, February 16, 1960)

Neurochemical transmitters such as catecholamines, serotonin and acetylcholine are known to be localized in intracellular granules of brain (*1–4*), and serotonin in those of duodenal mucosa (*5*). Furthermore, it was suggested by Hillarp *et al.* (*6*) that catecholamines exist in granules of adrenal medulla in a bound form with some high-molecular compound. It is now generally believed that neurochemical transmitters in brain granules can exist in either physiologically active or inactve form depending upon physiological conditions (*7*).

Platelets have been shown to be capable of accumulating serotonin (*8*) and catecholamines (*9*) from their media, and evidence has been obtained to indicate that this uptake is caused by an active transport mechanism (*8*, *9*). Attempts have also been made to determine if these transmitters exist in platelets in a bound form, however none of these compounds could be demonstrated in such a form (*9*, *10*). It is probable that the transmitters in this case are accumulated by an active transport mechanism without involving the formation of firm linkages.

The purpose of the present paper is to describe the results of an investigation on the accumulation of noradrenaline in guinea pig brain mitochondrial particles.

MATERIALS AND METHODS

Mitochondrial Particles—Brain, liver and kidney mitochondrial particles were prepared from decapitated guinea pips according to the method of Hogeboom *et al.* (*11*) except for the following modifications; centrifugation at 600×*g* for 10 minutes was applied to remove cell debris and that at 10,000×*g* for 10 minutes to isolate the mitochondria. The particles were washed once and in the case of brain particles the fluffy layer formed on the bulk sediment was also used together with firmly sedimented mitochondrial particles. The pellets were kept at 0°C in an ice bath, and before use suspended in 0.25 *M* sucrose and adjusted to pH 6.8 with dilute sodium hydroxide.

Incubation Conditions—The standard incubation mixture contained 0.25 *M* sucrose, 5×10^{-3} *M* phosphate buffer at pH 6.8, mitochondrial particles (equivalent to 1.5 g. brain), 100 μg. or 2.36×10^{-4} *M* L-noradrenaline tartrate (neutralized with sodium hydroxide) in a total volume of 2.5 ml. The mixture was placed in a 12 ml.c entrifugal tube for Spinco model L ultracentrifuge and incubated for suitable period of time.

Fractionation of Incubation Mixture—After incubation, the mixture was placed in an ice bath for 5 minutes, diluted to 8.0 ml. with cold 0.25 *M* sucrose containing 5×10^{-3} *M* phosphate (pH 6.8), and centrifuged at 30,000×*g* for 10 minutes. The supernatant thus obtained was named Fraction S_1 (suspending medium). The pellets were washed twice with 9.0 ml. of 0.25 *M* sucrose containing 5×10^{-3} *M* phosphate (pH 6.8), and the combined washings were designated as Fraction S_2 (fraction washed out from particles). The washed pellets were suspended in the sucrose solution and named Fraction P (washed particles). All fractionation procedures were carried out at 0°C. Sucrose solution containing 5×10^{-3} *M* phosphate was used in the fractionation, since the fluffy particles are most readily sedimentable at this concentration of phosphate.

Determination of Noradrenaline—To 8.0 ml. of each fraction obtained as described above was added 2.0 ml. of 2 *N* perchloric acid and the mixture was allowed to stand for 10 minutes. Then, 0.6 ml. of 6 *N* potassium hydroxide was added to neutralize the mixture. The neutralized mixture was filtered and noradrenaline in a suitable portion of the filtrate was determined by the method of Weil-Marherbe (*12*). Five μg. of noradrenaline being used as the standard.

Estimation of Noradrenaline Concentrations—The concentrations of noradrenaline in the three fractions were calculated by dividing the quantity of noradrenaline found in each fraction by its volume. The volume of each fraction was estimated as follows. The volume of Fraction S_1 was taken as 2.5 ml. The volume of Fraction S_2 and that of Fraction P were taken as 80 per cent of the pellet volume measured by using a graduated pippet, assuming that the contaminating volume of extragranular fluid was 20 per cent of the pellet volume (*15*).

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Other Materials—Emanon 1112 (polyoxyethylene fatty acid ester) was supplied from Kao Soap Co. Ltd. Crystalline ATP* and DPN* were prepared as described previously (*13*). DOPA, adrenaline and 3-hydroxytyramine used were all L-form.

RESULTS

Recovery of Noradrenaline—The data recorded in Table I show that almost all of added noradrenaline could be recovered in the suspending medium (S_1), the washings (S_2) and the washed particles (P) after incubating the catechol derivative with brain mitochondrial particles. In fact, the recovery was almost quantitative (99–107 per cent). This fact indicates that the added noradreline does not undergo any metabolic alterations under the conditions employed, even though no inhibitors such as iproniazid were included in the incubation system. This conclusion received further support from the finding that noradrenaline could be recovered as such from Fraction P by means of chromatography on a Duolite C-25 column (*14*).

TABLE I

Recovery and Temperature Effect in Noradrenaline Accumulation by Guinea Pig Brain Mitochondrial Particles

Fraction	0°C			30°C		
	Experimental number					
	I	II	III	I	II	III
	µg.	µg.	µg.	µg.	µg.	µg.
S_1	85.9	85.1	89.5	87.2	84.9	95.1
S_2'	12.40	12.00	9.10	9.63	10.11	8.79
S_2''	1.66	1.51	1.66	1.96	2.34	1.40
S_2'''	0.37	0.43	0.29	0.46	0.46	0.34
P	0.34	0.56	0.70	1.31	1.58	1.73
S_2	14.43	13.94	11.05	12.05	12.91	10.53
Total	100.67	99.60	101.25	100.56	99.39	107.36

100 µg. or $0.76 \times 10^{-4} M$ noradrenaline was added to 8.0 ml. of reaction mixture. Incubation time, 20 minutes. After removing the medium (S_1), the pellets were washed three times with each 8.0 ml. of sucrose-phosphate solution to give three washings (S_2' S_2'' and S_2'''); the sum of which represents S_2. P is the washed pellets.

Two Types of Accumulation Mechanism—As shown in Table I, 0.3–0.7 per cent and 1.3–1.7 per cent of the added noradrenaline could be detected in Fraction P when incubation was conducted at 0°C and 30°C, respectively. Fraction S_2, on the other hand, contained 11–14 per cent of noradrenaline after incubation. (These data were corrected for the errors due to the contamination of supernatant in pellets, assuming that the contaminating volume was 20 per cent of the pellet volume (*15*)). It should be noted that the accumulation in Fraction S_2 is independent of the incubation temperature. It may be assumed from these results that noradrenaline is accumulated in the mitochondrial particles by two different mechanisms: (a) a temperature-dependent mechanism resulting in the accumulation of tightly bound noradrenaline (accumulation in Fraction P, or P-form accumulation), and (b) a temperature-independent process by which noradrenaline becomes only loosely bound to the particles (accumulation in Fraction S_2, or S_2-form accumulation).

Comparisons of Two Mechanism—The two accumulation mechanisms postulated above were found to differ further in several respects. Firstly, as is illustrated in Fig. 1, the S_2-form accumulation takes place immediately after mixing the particles with noradrenaline and thereafter becomes independent of incubation times. The P-form accumulation, on the other hand, is rather slow at the beginning, but gradually increases with increasing incubation times. Secondly, it was found that the two processes are quite different with regard to their stability to heat as shown in Table VI. Thus, pretreatment of the particles at 100°C for 25 minutes almost completely abolishes the accumulation in Fraction P, but the same treatment causes little effect on the S_2-form accumulation. These facts appear to suggest that the noradrenaline found in Fraction P has been accumulated through a certain enzymatic mechanism and that in Fraction S_2 has been simply adsorbed to the particles by a nonenzymatic mechanism.

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* The following abbreviations are used: ATP, adenosinetriphosphate; DPN, diphosphopyridine nucleotide; DOPA, 3.4-dihydroxyphenylalanine: DNP, 2,4-dinitrophenol.

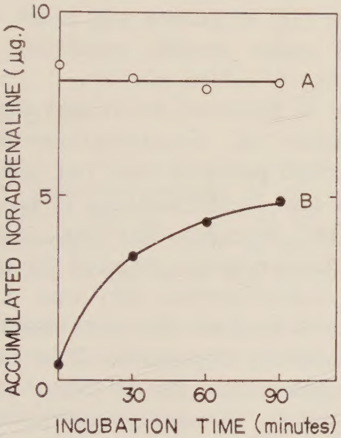

Fig. 1. Time course of noradrenaline accumulation. Curves A and B represent S_2 and P respectively.

Substrate and Tissue Specificities—It was found that other catecholamines than noradrenaline, such as adrenaline, DOPA, and 3-hydroxytyramine are also accumulated in the brain mitochondrial particles. Table II shows that the P-form accumulation of adrenaline and DOPA is significantly more pronounced than that of noradrenaline and 3-hydroxytyramine. The nonenzymatic adsorption (S_2-form accumulation), on the other hand, is most active with 3-hydroxytyramine and decreases with noradrenaline, adrenaline and DOPA in this order. This latter finding suggests that these catechol derivatives are adsorbed through some anionic sites on the surface of the particles.

Both of the two types of accumulation seem to be specific to brain mitochondrial particles, since, as shown in Table III, no accumulation of noradrenaline could be detected in both Fractions P and S_2 of mitochondrial particles prepared from liver and kidney.

Table II

Accumulation of Various Catecholamines in Brain Particles

Catecholderivative	S_2	P
	($\times 10^{-2}$ μmoles)	
Noradrenaline	4.14	1.16
Adrenaline	3.56	3.63
3-Hydroxytyramine	5.80	2.80
DOPA	2.03	3.59

100 μg. of catecholamine was added. Incubation was carried out at 30°C for 20 minutes.

Table III

Noradrenaline Accumulation in Brain, Liver and Kidney Mitochondrial Particles

Organ	S_1	S_2	P	Total
	μg.	μg.	μg.	μg.
Brain	87.1	7.35	4.05[1]	98.50
Liver	78.8	1.29	0.51	80.60
Kidney	100.7	1.12	0.64	102.46

Mitochondrial particles equivalent to 1.5 g. brain, 0.75 g. liver and 1.5 g. kidney were used. Incubation; 30°C, 20 minutes.

1) The higher value for P than those of Table I is due to the higher noradrenaline concentration used (*cf.* Table IX).

Nature of Accumulation Processes—In order to shed more light to the nature of the two types of accumulation mechanisms, the concentrations (but not absolute amounts) of noradrenaline and other catecholamines in Fraction S_1, S_2 and P were determined after incubating the brain particles with the catechol derivatives. The results of these experiments are recorded in Table IV in the forms of concentration ratios between P and

S_1 and between P and S_2. As will be seen, the ratio P to S_1 for noradrenaline exceeded 1.0 when incubation was continued for more than 60 minutes, and those for DOPA and adrenaline became higher than 1.0 after 20 minutes incubation. These findings indicating that the catecholamine accumulation from medium (S_1) into particles (P) can take place against concentration gradient provide a possibility for the operation of an enzymatic transport mechanism.

The ratio P to S_2, on the other hand, was always below 1.0 under the conditions employed. This is due to very hiph concentrations of catecholamines adsorbed to the surface of the particles (Fraction S_2). The low values obtained for P to S_2 ratio might suggest that the catechol derivatives are first adsorbed to the surface of the particles (Fraction S_2) and the adsorbed compounds then penetrate into the inside of the particles (Fraction P) by a simple diffusion process. This possibility seems, however, to be excluded, since if this is the case the ratio of P to S_2 obtainable for different catechol derivatives must not be so different as shown in Table IV. This inference is further supported by the data of Fig. 2 described in the later section.

It seems to be therefore more reasonable to conclude from these results as well as from those concerning temperature dependency, thermostability, and time course of the accumulation processes (seen above) that the accumulations into Fraction P and S_2 are independent processes and the P-form accumulation involves a certain enzymatic transport mechanism.

Relation to Energy Metabolism—An active transport mechanism usually requires a supply of energy from exergonic reactions, and, in fact, it has been reported that the accumulation of γ-aminobutyric acid (*16*) and noradrenaline (*17*) in guinea pig brain slices is enhanced under aerobic conditions and in the presence of added glucose.

Table V, however, shows that the P-form accumulation of noradrenaline in brain mitochondrial particles was not affected by aerobiosis and by the addition of glucose. It was further revealed that the addition of cofactors known to be required for glycolysis in brain mitochondria (*18*) was not only unable to stimulate the accumulation but also remarkably depressive. The possibility that endogenous glucose or other substrates are present in the mitochondrial particles employed could be eliminated from the data of Table VI indicating that the addition of glucose is not effective even after preincubat-

TABLE IV

Concentration Relations in Catecholamine Accumulation in Brain Particles

Experiment	Catecholamine	Incubation time (minutes)	Ratio of the concentration	
			P/S_1	P/S_2
I	Noradrenaline	30	0.96	0.13
	,,	60	1.16	0.17
	,,	90	1.39	0.19
II	Noradrenaline	20	0.51	0.13
	Adrenaline	,,	1.14	0.32
	3-Hydroxytyramine	,,	0.79	0.16
	DOPA	,,	1.43	0.57

After incubating brain mitochondrial particles with catechol derivatives, the concentrations (*M*) of the derivatives in Fraction S_1, S_2 and P were determined as described in Materials and Methods. The results are expressed as the concentration ratios P/S_1 and P/S_2. Incubation conditions of Experiments I and II were the same with those of Fig. 1 and Table II, respectively.

ing the particles at 30°C for 60 minutes. It is therefore considered that noradrenaline is accumulated in the particles without utilizing the energy generated from the catabolic breakdown of glucose.

TABLE V

Effect of Various Additions on Noradrenaline Accumulation in Brain Particles

Addition	Aerobic shaking	P
		μg.
None	−	2.68
Glucose	−	2.79
None	+	2.44
Glucose	+	2.82
ATP, $MgCl_2$, DPN and glucose	−	0.74

The final concentrations of additions were $1\times10^{-2}M$ for glucose, $5\times10^{-3}M$ for ATP, $1\times10^{-2}M$ for $MgCl_2$ and $2\times10^{-4}M$ for DPN. Incubation was carried out at 30°C for 20 minutes.

In agreement with this fact, the accumulation was not inhibited by cyanide and DPN which are well-known inhibitors of mitochondrial energy-generating systems (Table VIII, 1). Sodium and potassium fluoride, on the other hand, appeared to be appreciably inhibitory. It was, however, soon revealed that this inhibition is caused by the cations and that fluoride ion itself is rather slightly stimulatory (Table VIII, 1 and 2). A stimulatory effect of fluoride has also been observed in the incorporation of radioactive phosphorus into lipids of rat brain mitochondria (*19*). The inhibition by cations will be dealt with in some details in a later section.

Effects of Various Treatments—It is shown in Table VI that the P-form accumulation of noradrenaline is somewhat lowered by preincubating the particles at 30°C and by freezing and thawing. A more significant decrease in the accumulation was observed when the particles were kept under hypotonic conditions.

TABLE VI

Effect of Various Treatments on Noradrenaline Accumulation in Brain Particles

Experiment	Treatment	S_2	P
		μg.	μg.
I	None	8.20	3.36
	Preincubation (30°C, 30 min.)	7.71	2.85
	Preincubation (30°C, 60 min.)	8.24	2.81
	Preincubation (30°C, 30 min.) in 0.175 M sucrose1)	8.75	2.28
	Preincubation (100°C, 25 min.)	9.86	0.28
	Addition of glucose ($1\times10^{-2}M$) after preincubation (30°C, 60 min)	8.60	2.56
II	None		4.56
	Freezing and thawing2)		4.25
	0.05 M sucrose1)		3.20
	0.65 M sucrose1)		3.94

1) These conditions were constituted during incubation period.

2) Freezing and thawing was carried out five times in dry ice-ethanol.

The preincubation medium was same as standard incubation mixture.

Incubation was carried out at 30°C, for 20 minutes.

TABLE VII

Effect of Detergent on Noradrenaline Accumulation in Brain Particles

Emanon 1112	Optical density at 600 mμ of the suspension	S_2	P
%		μg.	μg.
—	1.33	7.21	3.09
0.05	1.25	7.12	2.90
0.1	1.23	7.80	3.34
0.3	0.62	5.80	0.36
0.6	0.43	5.68	0.30
1.0	0.37	5.27	0.18
2.0	0.32	4.27	0.20

Detergent was added at zero time of incubation. Incubation was carried out at 30°C, for 20 minutes.

As will be seen from Table VII, the nonionic detergent, Emanon 1112, exerted an interesting effect on the accumulation. The detergent exhibited no effect on the both types of accumulation up to 0.1 per cent, but at higher concentrations it caused considerable decrease in the S_2-form accumulation and at the same time completely inhibited the P-form accumulation.

It was also observed that the turbidity of the suspension expressed in terms of optical density at 600 mμ abruptly decreases at the detergent concentration of 0.3 per cent where marked reduction in noradrenaline accumulation begins. It may therefore be concluded that the mechanism of noradrenaline transport into the mitochondrial particles is closely dependent upon the intactness of particulate structures. The transport mechanism, however, seems to be more stable than that responsible for oxidative phosphorylation, since the latter mechanism in rat liver mitochondria has been shown to be completely inactivated by Emanon 1112 at concentrations causing no effect on the accumulation process (*20*). The stability of the mechanism is also supported by its resistance against aging (Table VI).

Inhibitory Actions of Cations—As mentioned in a previous section, the accumulation of noradrenaline in Fraction P and S_2 is inhibited by sodium and potassium ions. As shown in Table VIII, 3, other cations such as ammonium, magnesium and calcium are also inhibitory. In fact, divalent cations are more potent inhibitors than monovalent ones. That this inhibition is not due to the anions is indicated by the fact that sodium phosphate inhibited the process to the same extent as did the same concentration of sodium chloride.

It was found that the addition of 1×10^{-2} *M* divalent cations causes the aggregation of the particles. It is therefore possible that the inhibition by divalent cations is at least partly due to the degeneration of particulate structures caused by aggregation. Such aggregation was, however, not observed with mitochondrial suspensions prepared from liver and kidney.

At any rate, the fact that both the P- and S_2-form accumulations are inhibited by cations suggests that certain anionic groups of the mitochondrial particles are involved in these two processes. Actually, it has already

TABLE VIII

Effect of Various Inhibitors and Cations on Noradrenaline Accumulation in Brain Particles

Experiment	Addition	S_2		P	
		μg.	Per cent of inhibition	μg.	Per cent of inhibition
1	None	11.39		4.56	
	1×10^{-2} *M* KCl	8.01	30	2.69	41
	1×10^{-2} *M* KF			3.30	28
	5×10^{-5} *M* DNP			4.66	−2
	1×10^{-3} *M* KCN			4.10	10
2	None	7.21		3.09	
	1×10^{-2} *M* NaCl	6.19	14	1.92	38
	1×10^{-2} *M* NaF			2.34	24
3	None	7.35		4.05	
	1×10^{-2} *M* NaCl	6.31	14	2.54	37
	2×10^{-2} *M* NaCl	4.64	37	2.13	47
	1×10^{-2} *M* NH_4Cl	5.72	22	2.50	38
	5×10^{-3} *M* $MgCl_2$	3.93	46	1.77	56
	1×10^{-2} *M* $MgCl_2$	3.20	56	1.32	67
	5×10^{-3} *M* $CaCl_2$	3.05	58	1.42	65

Incubation was carried out at 30°C, for 20 minutes.

Table IX

Inhibition of Ammonium on Noradrenaline Accumulation in Brain Particles

NH_4Cl (*M*)	Initial noradrenaline concentration (*M*)	S_2		P	
		μ moles	Per cent of inhibition	μ moles	Per cent of inhibition
—	0.628×10^{-3}	0.137		0.052	
	1.256 ,,	0.276		0.101	
	1.882 ,,	0.412		0.154	
	3.762 ,,	0.854		0.338	
	12.55 ,,	2.475		0.938	
1.33×10^{-2} *M* NH_4Cl	0.628×10^{-3}	0.121	11.7	0.043	17.8
	1.256 ,,	0.229	17.0	0.089	11.9
	1.882 ,,	0.326	20.7	0.136	11.7
	3.762 ,,	0.742	13.1	0.296	12.4
	12.55 ,,	2.155	13.0	0.881	6.1

Incubation was carried out under the condition supplying NaCl until the concentration of sodium in the medium became 1.24×10^{-2} *M* and of excluding phosphate buffer. Incubation; 30°C, 20 minutes.

been assumed that in the S_2-form accumulation noradrenaline combines with some anionic sites on the surface of mitochondrial particles.

If mitochondrial anionic groups are actually involved in the accumulation processes, it is expected that the inhibition by cations should be competitive in nature. This expectation was confirmed in the case of P-form accumulation as shown in Table IX which indicates that the process is competitively inhibited by ammonium ion, a monovalent cation. Quite contrary to expectation, however, no such competition could be demonstrated in the case of S_2-form accumulation. This fact was also observed in the case of inhibition by sodium. As shown in Fig. 2, A, the decrease of the ratio S_2 to S with increasing the initial concentration of noradrenaline indicates that the inhibition by sodium of noradrenaline tartrate in the case of S_2-form accumulation is not competivively, and, on the other hand, no such decrease of the ratio P to S indicates that the inhibition in P-form accumulation is competitively. Under the condition of supplying sodium at a concentration of 1.24×10^{-2} *M* in the medium, as shown in Fig. 2, B, no change of the ratio S_2 to S was observed.

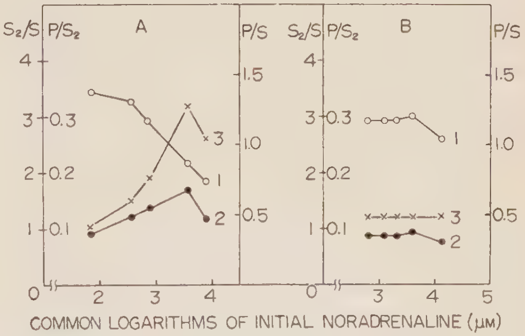

Fig. 2. Inhibition of sodium (of noradrenaline tartrate) an noradrenaline accumulation in brain particles.

S represents initial noradrenaline concentration (*M*). The concentrations (*M*) of noradrenaline in Fraction S_2 and P were determined as described in Materials and Methods.

Curve 1, 2 and 3 of A represent respectively the ratio S_2/S, P/S and P/S_2 under the condition without supplying sodium and containing 5×10^{-3} *M* phosphate buffer (0.85×10^{-2} *M* cation). Curves of B represent the above ratios under the condition supplying NaCl until the concentration of sodium in the medium became 1.24×10^{-2} *M* and excluding phosphate buffer. Incubation; 30°C, 20 minutes.

As mentioned in a previous section, the possibility that noradrenaline penetrate into the inside of the particles by a simple diffusion process was eliminated from the data of Fig. 2, A indicating that the P-form accumulation (P/S) is not in parallel with the S_2-form accumulation (S_2/S).

DISCUSSION

Although it is of considerable interest to elucidate the nature of anionic substances involved in the transport of noradrenaline into the mitochondrial particles (P-form accumulation), there is not yet sufficient evidence available to permit any decisive conclusions. It is, however, tempting to assume that structural components of mitochondrial particles, especially phospholipids, are the substances responsible for this function. This possibility may be inferred from the fact that the transport mechanism is closely dependent on the intactness of particulate structures. The finding that noradrenaline transport can be detected only with brain mitochondrial particles which are rich in phospholipids and not with liver and kidney particles containing relatively small amount of lipid components also lends some, though indirect, support for this inference.

If the phospholipid is actually involved in transport mechanism (*21*), it is expected that the P-form accumulation of noradrenaline is coupled with the energy-generating systems such as glycolysis and oxidative phosphorylation (*19*, *22*). It was, however, found that noradrenaline is accumulated into the particles without utilizing the energy derived from these systems. Whatever the explanation of the observations which have been described, a thorough investigation of the inhibitory actions of cations appears to be necessary.

SUMMARY

1. Mitochondria particles prepared from guinea pig brain can accumulate noradrenaline by two different mechanisms. While one mechanism involves a nonenzymatic adsorption of the catecholamine to certain anionic sites existing on the mitochondrial particles, the other mechanism represents probably an enzymatic transport of the catecholamine into the inside of the particles. Both mechanisms seem to be specific to brain mitochondrial particles and could not be observed with liver and kidney particles.

2. The transport mechanism is not inhibited by cyanide, 2,4-dinitrophenol and fluoride. This fact suggests that the process is not coupled with such energy-generating systems as glycolysis and oxidative phosphorylation.

3. The transport mechanism appears to be more stable than that responsible for oxidative phosphorylation, but is closely dependent on the intactness of particulate structures.

4. The transport is competitively inhibited by cations. It is therefore probable that certain anionic groups of the mitochondrial particles are involved in the transport mechanism.

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Über das Vorkommen der Freien Aminosäuren in Verschiedenen Rinderorganen

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Da die chemischen Stoffe, aus welchen sich die Zelle zusammensetzt, einerseits die Grundlage der in der Zelle verlaufenden chemischen Vorgänge bilden und andererseits die Organisationsanlage bei den verschiedenen tierischen Körpers begründen, so verlohnt es der Mühe, die chemische Zusammensetzung der einzelnen Organe bei verschiedenen Tierarten vergleichend zu untersuchen.

Nachdem schon Tomita und seine Mitarbeiter (*1*) aus Thymus-, Pankreas-, Muskel- und Hautgeweben von verschiedenen Tieren neue organische Verbindungen isoliert haben, habe ich nunmehr weitere chemische Untersuchungen der Rinderorgane unternommen und speziell meine Aufmerksamkeit den freien Aminosäuren geschenkt.

Vor mehreren Jahren haben Tomita (*2*) und Fukuda (*3*) neben verschiedenen Aminosäuren Spermin, Spermidin, Uracil, Bernsteinsäure und drei verschiedene Verbindungen, die die Bruttoformeln $C_8H_{18}O_4N_4$, $C_{19}H_{39}O_9N_4$ und $C_{20}H_{41}O_9N_4$ respektive haben, aus Schweinepankreas kristallinisch isoliert. Um die Anwesenheit dieser unbekannten Substanzen in Pankreas der anderen Tierarten zu vermitteln, haben Tsunoo, Musashi und Horisaka (*4*) neulich gefrorenes Walpankreas behandelt und das Ophidin kristallinisch dargestellt, das bis jetzt nur aus den Schlangenmuskeln abgetrennt wurde.

Nachdem durch voranstehende Untersuchungen festgestellt wurde, dass die Extraktivstoffe des Schweinepankreas in ihrer Zusammensetzung eine Abweichung von denen des Wals zeigten, lag es nahe, nun auch das Pankreas der anderen Arten von Säugetieren einen näheren Studium zu unterziehen.

Von diesem Gesichtspunkt ausgehend, habe ich die vergleichende Untersuchung der chemischen Zusammensetzung der Rinderpankreasdrüse unternommen.

Aus dem Rinderpankreas wurden Histidin, Lysin, Tryptophan und Tyrosin kristallinisch dargestellt. Als Monoaminosäuren wurden weiter Asparaginsäure, Glutaminsäure, Threonin, Cystin, Serin, Glycin, Alanin, Prolin, Tyrosin, Valin, Tryptophan, Leucin und Phenylalanin papierchromatographisch nachgewiesen.

Auf dem Papierchromatogramm zeigte sich, dass β-Alanin in Rinderpankreas in freier Form vorkommt. Die Anwesenheit des ungebundenen β-Alanin und γ-Aminobuttersäure ist auch in den Hypophysen und Herzmuskeln des Rindes erwiesen.

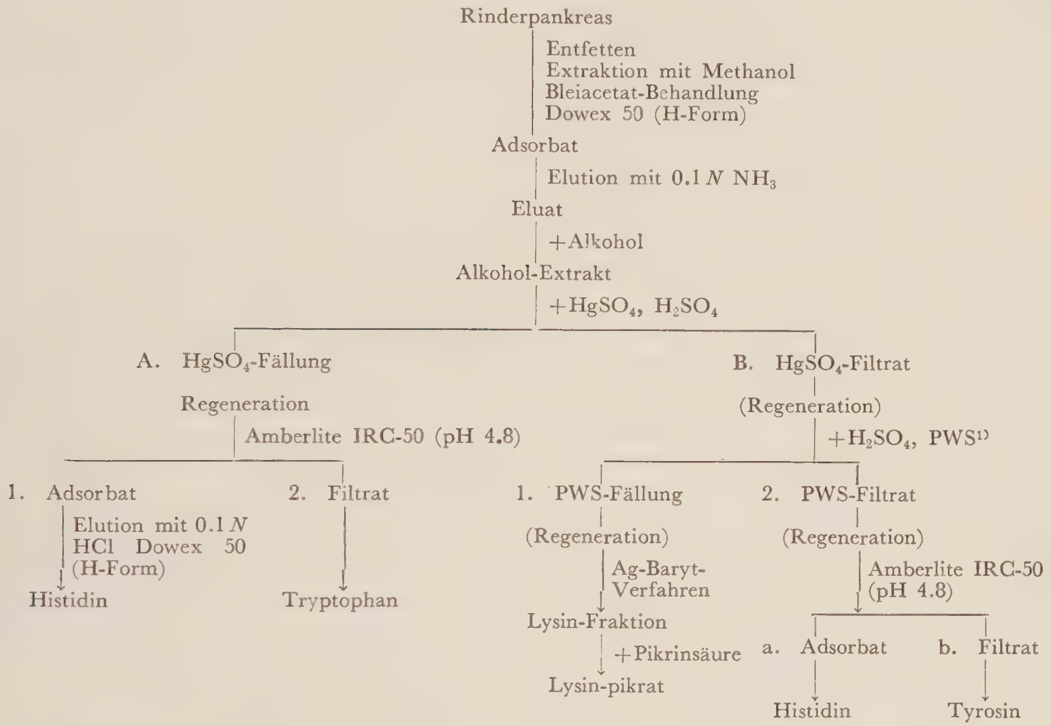
BESCHREIBUNG DER VERSUCHE

I. Darstellung der freien Aminosäuren in kristallinen Formen aus Rinderpankreas

Siebenhundert fünf und siebzig Rinderpankreas (etwa 165 kg.), die von Schlachthofe möglichst frisch und schnell bezogen wurden, wurden fein zerkleinert, mit Aether behandelt und dann mit Methanol (50°C) extrahiert. Wurde nun der Alkohol *in vacuo* abgedampft, hat man die anorganischen Salze, Proteine und Inosit mit Bleiacetatlösung möglichst beseitigt. Das Filtrat wurde mit Schwefelwasserstoff zerlegt. Die von Schwefelblei abfiltrierte Lösung wurde nach dem Entfernen des Schwefelwasserstoffs durch eine Säule mit Dowex 50 (H-Form) geschickt. Anschliessend wurde die Säule mit Wasser gespült. Die adsorbierten Stoffe hat man dann durch 0.1 *N* Ammoniaklösung eluiert. Die Eluat-Fraktion wurde *in vacuo* zum Sirup gebracht, mit Alkohol ausgezogen und nach der Beseitigung des

Alkohols in Wasser gelöst. Die so entstandene wässrige Lösung wurde mit $HgSO_4$ (in 5% H_2SO_4) gefällt. Die Fällung wurde als A auf Histidin und Tryptophan und das Filtrat als B auf Lysin, Histidin und Tyrosin nach dem Schema (Schema I) verarbeitet.

SCHEMA I



1) Phosphorwolframsäure

A. $HgSO_4$-Fällung—Nach der Zerlegung durch H_2S wurde das Schwefelquecksilber abfiltriert. Das Filtrat wurde nach dem Entfernen des Schwefelwasserstoffs ferner durch Dowex 50 (H-Form) behandlet. Anschliessend wurde die Säule mit Wasser gespült, das Adsorbat mit 0.1 N NH_3-Lösung eluiert. Diese Eluat-Fraktion wurde durch eine Säule mit Amberlite IRC-50 zu PH 4.8 kontrolliert, geschickt.

1. Adsorbat—Die Säule wurde mit 0.1 N HCl eluiert. Die Eluat-Fraktion wurde ferner durch Dowex 50 (H-Form) behandelt. Durch Wiederholung der Adsorption und Eluation hat man endlich eine kristallinische Substanz erhalten, die sich auf dem Papierchromatogramm zeigte, dass die erhaltene Verbindung den gleichen R_f-Wert besass, wie L-Histidin. (Ausbeute 0.2 g.)

TABELLE I

R_f-Wert

Verbindung	R_f in				
	Et	Bu/Ac	Py	Py/Bu	Phen
Isolierte Subst.	0.05	0.10	0.50	0.14	0.64
Histidin	0.05	0.10	0.50	0.14	0.64

Versuchsbedingungen. Toyo-Filtrierpapier Nr. 51; aufsteigende Methode.
Lösungsmittel
Et (95% Alkohol);
Bu/Ac (Buthanol: Eisessig: Wasser 80:20:20, frisch bereitet);
Py (Pyridin: Wasser 65:35);
Py/Bu (Pyridin: Buthanol: Wasser 80:20:20);
Phen (Phenol, gessättigt mit einer wässrigen Lösung von 6.3% Na-Citrat und 3.7% KH_2PO_4).

2. *Filtrat*—Wurde die mit Amberlite IRC-50 nicht adsorbierte Lösung eingeengt, so schieden sich allmählich am Boden weisse kristallinische Niederschläge aus. Nach dem Umkristallisieren aus wässrigem Alkohol bekam man 2.5 g. Kristalle in Blättchen, deren Eigenschaften, Analysenzahlen und papierchromatographische R_f-Werte auf Tryptophan passten.

$C_{11}H_{12}O_2N_2$
Ber. C 64.69 H 5.92 O 15.67 N 13.72
Gef. 64.20 6.02 15.67 13.36
64.56 5.87 15.46 —

Adamkiewitz's Reaktion und Ninhydrin-Färbung sind stark positiv.
Schmp. 273°C. $[\alpha]_D^{29}=-32.28°$ (in H_2O)
Tabelle II zeigt papierchromatographischen Vergleich der R_f-Werte der erhaltenen Substanz und des Tryptophans.

TABELLE II

R_f-Wert

Verbindung	R_f in					
	Et	Met	Bu/Ac	Py	Py/Bu	Phen
Isolierte Subst.	0.11	0.31	0.41	0.70	0.48	0.75
Tryptophan	0.11	0.31	0.43	0.70	0.50	0.79

Met (95% Methanol)

B. $HgSO_4$-Filtrat—Das Filtrat wurde mit H_2S zerlegt. Das von Schwefelquecksilber abfiltrierte Lösung wurde mit Phosphorwolframsäure (PWS) in 5% H_2SO_4 gefällt.

1. PWS-Fällung—Der Niederschlag wurde mit Baryt zerlegt, mit CO_2 behandelt und der Bariumrest mit H_2SO_4 beseitigt. Die Lösung wurde mit Dowex 50 (H-Form) behandelt. Nach der Elution mit 0.1 *N* NH_3 wurde die Lysin-Fraktion nach dem Kossel-Kutscherschen Silber-Baryt-Verfahren hergestellt. Die Silberverbindung der Lysin-Fraktion wurde mit verd. H_2SO_4 verrührt und mit H_2S behandlet. Das Filtrat vom Schwefelsilber wurde eingeengt und die Schwefelsäure mit Baryt beseitigt. Dann wurde die Lösung ferner mit den oben gebrauchten Ionenaustausch-Harzen gereinigt, und endlich mit Pikrinsäurelösung versetzt. Daraus schieden sich 0.7 g. gelbe, nadelförmige Kristalle aus, die einen Schmelzpunkt von 253°C (unter Zers.) besitzen.

$C_6H_{14}O_2N_2 \cdot C_6H_3O_7N_3$
Ber. C 38.40 H 4.57 N 18.66
Gef. 38.18 4.82 18.43

Nach der folgenden Papierchromatographie erwies sich diese Substanz als Lysin.

TABELLE III

R_f-Wert

Verbinding	R_f in				
	Et	Bu/Ac	Py/Bu	Py	Phen
Isolierte Subst.	0.05	0.10	0.05	0.20	0.45
Lysin	0.05	0.09	0.05	0.20	0.45

Damit ist das Vorkommen von Lysin in Rinderpankreas festgestellt.

2. *PWS-Filtrat*—Das Filtrat wurde mit Baryt zerlegt, mit verd. H_2SO_4 von Barium befreit und dann durch eine Säule mit Dowex 50 (H-Form) geschickt. Nach der Wasserspülung und Elution durch 0.1 *N* NH_3-Lösung wurde das Eluat mit Amberlite IRC-50, zu pH 4.8 kontrolliert, behandelt. Man hat dann das Adsorbat a und das Filtrat b verteilt.

a. Adsorbat—Die Säule wurde nach der Wasserspülung mit 0.1 *N* HCl eluiert. Die Eluat-Fraktion wurde ferner durch Dowex 50 (H-Form) behandlet. Nach der Wasserspülung und Elution durch 0.1 *N* NH_3-Lösung wurde das Eluat im Vak. unter der Hinfügung des Wassers mehrmals eingeengt, um überschüssige Ammoniak zu entfernen. Wurde die im Boden ausgeschiedenen Kristalle mit wässrigem Alkohol umkristallisiert, so wurden 0.7 g. nadelförmige Kristalle erhalten, die bei 268°C (unter Zers.) schmolzen. Die wässrige Lösung reagiert schwach basisch. Ninhydrinfärbung und Pauly's Diazoreaktion sind stark positiv. $[\alpha]_D^{25}=-41.68°$.

$C_6H_9O_2N_3$
Ber. C 46.44 H 5.85 O 20.62 N 27.08
Gef. 46.53 5.95 20.79 26.87
46.44 5.88 21.06 26.95

Aus den Analysenzahlen und dem Drehungsvermögen geht hervor, dass die Substanz aus L-Histidin bestand.

TABELLE IV

R_f-Wert

Verbindung	R_f in				
	Et	Bu/Ac	Py	Py/Bu	Phen
Isolierte Subst.	0.05	0.10	0.50	0.14	0.64
Histidin	0.05	0.10	0.50	0.14	0.64

b. Filtrat—Die durch eine Säule von Amberlite IRC-50 nicht adsorbierte Lösung wurde unter vermindertem Druck stark eingeengt. Dabei schieden sich seidenartige nadelförmige Kristalle aus (0.9 g.), die nach Umkristallisieren mit heissem Wasser bei 285°C (unter Zers.) schmolzen. Millon's Probe war stark positiv.

$C_9H_{11}O_3N$
Ber. C 59.66 H 6.12 N 7.73
Gef. 59.55 6.15 7.45

Es unterliegt keinem Zweifel, dass die hier erhaltene Substanz mit dem Tyrosin identisch war.

TABELLE V

R_f-Wert

Verbindung	R_f in	
	Bu/Ac	Py/Bu
Isolierte Subst.	0.38	0.48
Tyrosin	0.38	0.48

Man konnte also 4 Arten von Aminosäuren, nämlich Lysin, Histidin, Tryptophan und Tyrosin aus Rinderpankreas kristallinisch dargestellt.

II. Papierchromatographische Untersuchung der Monoaminosäuren in dem Rinderpankreas

In der mit Amberlite IRC-50 nicht adsorbierten und durch Phosphorwolframsäure nicht fällbaren Fraktion (b. Filtrat in Schema I) vom Rinderpankreas müssen noch verschiedene Monoaminosäuren enthalten sein; da meine Bemühungen erfolglos gewesen sind, sie zu einem definitiven kristallisierten Stoff zu kommen, so habe ich diese Fraktion mit Dowex 50 (H-Form) behandelt. Das Adsorbat wurde nach Partridge'scher Methode (*5*) mit 0.1 *N* NH_3-Lösung eluiert. Das Eluat wurde in 31 Fraktionen von je 15 ml. verteilt, und die R_f-Werte jeder Fraktion in verschiedenen Lösungsmitteln bei verschiedenen Bedingungen bestimmt und mit natürlichen Aminosäuren verglichen. Die Ergebnisse der papierchromatographischen Untersuchungen zeigt Tab. VI.

TABELLE VI

Papierchromatographische Untersuchung des Rinderpankreas

Verteilung d. Fraktionsammlers	R_f in Lös. mittel		Identifizierte Substanz
	Bu/Ac	Py/Bu	
I (1~10)	0.19	0.04	Asparaginsäure
	0.18	0.18	Serin
	0.27	0.10	Glutaminsäure
	0.26	0.28	Threonin
II (11~24)	0.06	0.60	Cystin
	0.21	0.14	Glycin
	0.32	0.28	Alanin
	0.37	—	Prolin
	0.38	0.48	Tyrosin
	0.49	0.46	Valin
	0.48	0.50	Tryptophan
	0.64	0.60	Leucin
III (25~31)	0.06	0.06	Cystin
	0.21	—	Glycin
	0.30	0.29	Alanin
	0.33	0.14	β-Alanin
	0.38	—	Tyrosin
	0.49	0.46	Valin
	0.48	0.50	Tryptophan
	0.60	—	Phenylalanin
	0.64	0.60	Leucin

III. Papierchromatographischer Nachweis von ω-Aminosäuren in Verschiedenen Rinderorganen

Wie bei dem Pankreas, so wurden Hypophysen und Herzmuskeln möglichst frisch zur

Untersuchungen geliefert. Extraktion, Entfetten und Enteiweissung wurden ähnlich wie beim Pankreas behandelt, die regenerierte Lösung zunächst durch eine Säule mit Dowex 50 (H-Form) gereinigt. Zuletzt hat man die Lösung mit Amberlite IRC-50, zu pH 4.8 kontrolliert behandelt. Die durch eine Säule dieser Ionenaustauscher nicht adsorbierte Lösung wurde wieder mit Dowex 50 (H-Form) behandelt. Das Adsorbat wurde nach Partridge'scher Methode (*5*) mit 0.1 N NH_3-Lösung eluiert. Das Eluat in vielen Fraktionen verteilt, und papierchromatographisch Untersucht. Dabei in letzten Fraktionen habe ich ω-Aminosäuren bemerkt. Die Ergebnisse der papierchromatographischer Untersuchung zeigt Tab. VII.

TABELLE VII

Papierchromatographischer Vergleich der ω-Aminosäuren

Rinderorgane	R_f in						Identifizierte ω-Aminosäuren
	Bu/Ac		Py/Bu		Phen		
Pankreas	0.33		0.14				β-Alanin
Hypophyse	0.34	0.43	0.15	0.17			β-Alanin und γ-Aminobuttersäure
Herzmuskel	0.31	0.41	0.14	0.15	0.64	0.74	β-Alanin und γ-Aminobuttersäure
β-Alanin	0.32		0.14		0.64		
γ-Aminobuttersäure		0.42		0.16		0.74	

Zusammenfassung

1. Aus dem Rinderpankreas wurden Histidin, Lysin, Tryptophan und Tyrosin kristallinisch dargestellt.

2. Als Monoaminosäuren wurden weiter Asparaginsäure, Glutaminsäure, Threonin, Cystin, Serin, Glycin, Alanin, Prolin, Tyrosin, Valin, Tryptophan, Leucin and Phenylalanin papierchromatographisch nachgewieson.

3. Auf dem Papierchromatogramm zeigte sich, dass β-Alanin in Rinderpankreas in freier Form vorkommt. Die Anwesenheit des ungebundenen β-Alanin und γ-Aminobuttersäure ist auch in den Hypophysen und Herzmuskeln des Rindes erwiesen.

Diese Arbeit wurde zum Teil mit Mitteln ausgeführt, die Herrn Prof. M. Tomita von dem Waksman-Stipendium bewilligt worden war, wofür wir denselben zu aufrichtigem Danke verpflichtet sind.

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Studies on Formate Activation

I. Purification and Properties of Formokinase

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Much literature has appeared in recent years on formate activation. Greenberg *et al.* (*1*) using pigeon liver first provided evidence for the ATP*-dependent activation of formate with the participation of THFA, according to the following equation:

$$\mathrm{HCOOH+THFA+ATP} \rightleftharpoons \mathrm{f^{10}THFA+ADP}+i\mathrm{P}$$

where f^{10} represents formate combined with carbon 10 of THFA. Recently a formate activating enzyme (tetrahydrofolic formylase) has been purified from pigeon liver and various kinds of bacteria, and its mode of action extensively studied. Using a highly purified enzyme from *Micrococcus aerogenes*, Whiteley *et al.* (*2*) obtained evidence that the activation mechanism consisted of two kinds of reactions, the first, the interaction of ATP and THFA to form a phosphorylated coenzyme and the second, the reaction of the phosphorylated coenzyme with formate to yield f^{10}THFA.

Recent work on fatty acid activation demonstrated the activation of the carboxyl group, forming acy-phosphate in some bacteria, and acyl-AMP in animals and in yeast. This suggests that, by analogy with the mechanism of fatty acid activation, formate can be activated without the participation of THFA.

In a preliminary note (*3*), we reported the isolation of a new formate activating enzyme from *E coli*. and some of its properties. Our enzyme has an entirely different formate activation mechanism from that previously reported by Whiteley *et al.* (*2*). Therefore, from the reaction mechanism, and to avoid confusion, the authors proposed the name, formokinase, for the enzyme.

The present paper describes the properties of formokinase and the reaction product.

EXPERIMENTAL

Materials—ATP and AMP were obtained from Nutritional Biochemicals Co., GTP and UTP from Sigma Chemical Co., and formic acid from Merck Co. ADP was prepared by the method of Chamber *et al.* (*4*). Formohydroxamic acid was prepared from ethyl formate and hydroxylamine by a modification of the method of Safir *et al.* (*5*). Formyl phosphate was synthesized from dibenzylphosphochloridate and anhydrous formic acid (*6*). Other chemical compounds were purchased from commercial sources in Japan.

Analytical Procedures—Formokinase activity was measured colorimetrically by the method of Schweet (*7*). The assay system consisted of 600 μmoles of potassium formate, 10 μmoles of ATP, 6 μmoles of 2-mercaptoethanol, 20 μmoles of $MnCl_2$, 2 mmoles of hydroxylamine (neutralized with KOH), 150 μmoles of Tris buffer, pH 7.2, enzyme and water to a final volume of 3.0 ml. Incubation was carried out at 37°C for 20 minutes. 1 unit is defined as the amount of enzyme catalyzing the formation of 1.0 μmole of formohydroxamic acid per 60 minutes.

Acetokinase activity was measured by the same assay system as that for formokinase except that manganese was replaced by magnesium. Incubations were at 37°C for 5 minutes. 1 unit of acetokinase is defined as the amount which forms 1.0 μmole of acetohydroxamic acid per minutes.

ATP was determined by the acetokinase method (*8*) and other adenine nucleotides were measured by the procedure of Cohn (*9*). Inorganic orthophosphate and pyrophosphate were estimated by the methods of Fiske and SubbaRow (*10*) and of Flynn *et al.* (*11*) respectively.

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* The abbreviations used here are: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine 5′-phosphate; GTP, guanosine triphosphate; UTP, uridine triphosphate; *i*P, inorganic orthophosphate; THFA, tetrahydrofolic acid; f^{10}THFA, N^{10}-formyl tetrahydrofolic acid; CoA, coenzyme A; EDTA, ethylenediaminetetraacetic acid; Tris buffer, tris(hydroxymethyl)aminomethane-HCl buffer.

Protein was measured by the method of Lowry *et al.* (*12*) and by the modified Biuret reaction of Pardee*.

Formohydroxamic acid on paper chromatograms was detected and identified by spraying the papers with 5% $FeCl_3$ in $0.1\,N$ HCl. Paper chromatography of nucleotides was carried out by the procedure of Magasanik *et al.* (*13*) and the nucleotides were detected under a mineral light lamp.

Cultivation of Organisms—E. coli, strain B, was grown at 37°C in a medium with the following composition: 5 g. of glucose, 1 g. of sodium formate, 2.5 g. of peptone, 0.5 g. of yeast extract, 2.5 g. of $(NH_4)_2SO_4$, 2 g. of KH_2PO_4, 3 g. of Na_2HPO_4, 2 g. of NaCl, 0.5 g. of $MgSO_4 \cdot 7H_2O$ and tap water to 1 liter (pH 7.2). After 16 hours, the cells were harvested and acetone-dried.

RESULTS

Purification of Formokinase

Step I, Crude Extract—All purification procedures were carried out at 0°C, unless otherwise noted. Ten grams of the acetone-dried cells were extracted with 200 ml. of cold distilled water for 16 hours in a refrigerator. After centrifugation, 1 M Tris buffer, pH 7.2, was added to the supernatant to a final concentration of 0.05 M.

Step II, First Acetone Fractionation—To 160 ml. of the crude extract, acetone was added slowly at −10°C to a final concentration of 45 per cent. The resultant precipitate was removed by centrifugation. The acetone concentration was increased to 60 per cent, and the precipitate collected by centrifugation and dissolved in 80 ml. of 0.02 M potassium phosphate buffer, pH 7.2.

Step III, Calcium Phosphate Gel Treatment—Enzyme from Step II was mixed with calcium phosphate gel (1 mg. of gel per mg. of protein) and stirred mechanically for 15 minutes. After centrifugation, the precipitate was discarded and the supernatant purified further.

Step IV, Ammonium Sulphate Fractionation—Powdered ammonium sulfate was slowly added with mechanical stirring to 0.5 saturation. After centrifugation, the ammonium sulfate concentration was increased to 0.65 saturation. The precipitate was collected by centrifugation and suspended in 0.65 saturated ammonium sulfate solution, previously adjusted to pH 7.2 with ammonia.

The suspension was then applied to a cellulose column which had been washed by 0.65 saturated ammonium sulfate solution. The column was eluted in turn by 100 ml. each of 0.58, 0.53 and finally 0.50 saturated ammonium sulfate solution. The fraction eluted at 0.53 saturation was precipitated by adding sufficient solid ammonium sulfate and the resultant precipitate was dissolved in 0.05 M Tris buffer, pH 7.2, containing 0.002 M 2-mercaptoethanol. Most of the formokinase activity was found in this fraction.

Step V, Second Acetone Fractionation—Step IV enzyme was fractionated with between 50 and 55 per cent acetone at −10°C. The precipitate was dissolved in 0.05 M Tris buffer, pH 7.2, containing 0.002 M 2-mercaptoethanol.

These purification procedures are summarized in Table I. The specific activity of formokinase usually increased 50 to 60-fold during purification. The purified enzyme can be stored at −20°C for over one week without significant loss of activity.

Table I

Purification of Formokinase

Purification step	Protein (mg.)	Total units	Specific activity[1]	Yield (per cent)
I Crude extract	2,443	5,590	2.28	100
II 1st Acetone fractionation	1,398	5,390	4.16	98
III Calcium phosphate gel treatment	1,186	5,190	4.50	95
IV Ammonium sulfate fractionation	70.2	2,820	40.2	54
V 2nd Acetone fractionation	14.9	1,950	131.0	35

1) Units per mg. of protein.

Cofactor Requirements—As shown in Table II, the activation of formate is strikingly dependent upon ATP. GTP can partially substitute for ATP, but other nucleotides tested were all without effect (see Table III). The role of GTP is not clear at present.

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* Pardee, A. B., personal communication.

From Table II, it is clear that the formokinase activity is absolutely dependent upon the presence of manganese. The optimal concentration of this cation was approximately $6.67 \times 10^{-3} M$ (Fig. 1). Of the other divalent cations tested, only magnesium could substitute for manganese but it was much less effective.

Table II

Cofactor Requirements for Formate Activation

Reaction system	Hydroxamate formed (μmoles per 20 minutes)
Complete system	1.76
-ATP	0
-$MnCl_2$	0.06
-$MnCl_2$+$MgCl_2$	0.46
-glutathione	0.36
-glutathione+L-cysteine (5 μmoles)	1.27
-glutathione+thioglycolate (5 μmoles)	0.94

The complete system contained: 600 μmoles of potassium formate, 15 μmoles of ATP, 3 μmoles of reduced glutathione, 20 μmoles of $MnCl_2$, 2 mmoles of hydroxylamine (neutralized with KOH), 150 μmoles of Tris buffer, pH 7.2, 136 μg. of formokinase (Step V) and water to a final volume of 3 ml. All incubations were carried out at 37°C for 20 minutes.

Table III

Effect of Various Nucleotides on Formokinase Activity

Experiment No.	Nucleotide added	Hydroxamate formed (μmoles 20 minutes)
1	ATP	1.04
	GTP	0.73
	UTP	0.23
2	ATP	1.76
	ADP	0.18
	AMP	0.03

Nine micromoles of each nucleotide were used in Experiment 1 and 15 μmoles in Experiment 2. Other conditions were the same as described in the text.

During purification, especially at Step IV, considerable loss of activity was observed. This inactivation, however, could be reversed by addition of both ATP and manganese if reduced glutathione was also added. The

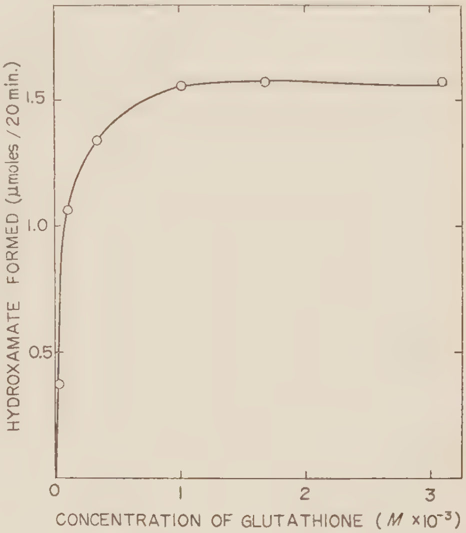

Fig. 1. Saturation curve for reduced glutathione. The conditions were as described in the text except that glutathione was substituted for 2-mercaptoethanol. Step IV enzyme (348 μg. of protein) was used.

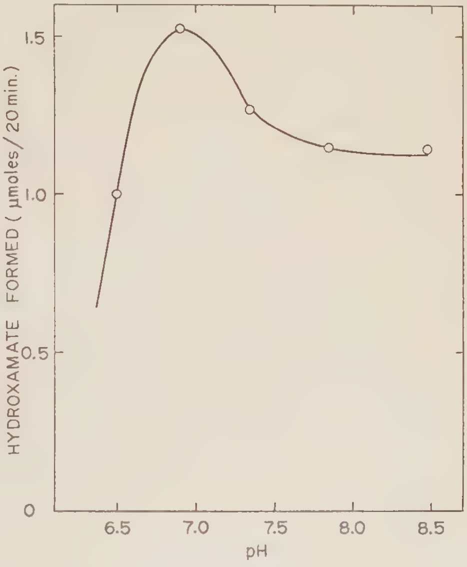

Fig. 2. pH-dependence of formokinase. Step IV enzyme (348 μg. of protein) was used. For conditions see text.

saturation curve for reduced glutathione is given in Fig. 2, showing that saturation occurs at a final concentration of $1 \times 10^{-3} M$. Cysteine, thioglycolate and 2-mercaptoethanol could also replace glutathione.

Treatment of the enzyme by charcoal or Dowex-1 (Cl^- form) (*14*) caused no appreciable loss of activity. This means that neither CoA nor THFA participates in the formokinase reaction.

pH Optimum—As shown in Fig. 3, the pH range of formokinase activity is wide, extending from pH 6.7 to 8.5 with an optimum at about 6.9 in Tris buffer.

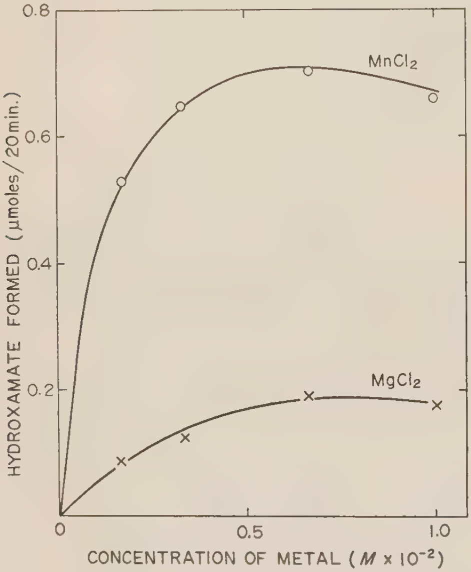

FIG. 3. Effect of metal concentration on the reaction velocity. Step IV enzyme (174 μg. of protein) was used. For conditions see text.

Identification of the Reaction Product—To determine the product of the formokinase reaction in the presence of hydroxylamine, a large scale experiment was conducted. The reaction mixture consisted of 6 mmoles of potassium formate, 150 μmoles of ATP, 30 μmoles of reduced glutathione, 200 μmoles of $MnCl_2$, 20 mmoles of hydroxylamine (neutralized with KOH), 1.5 mmoles of Tris buffer, pH 7.2, 1.36 mg. of formokinase (Step V) and water to a final volume of 30 ml. Incubation was carried out at 37°C for 1 hour.

The reaction was stopped by adding perchloric acid to a final concentration of 5 per cent. After centrifugation and neutralization with KOH, the supernatant was evaporated to dryness under reduced pressure. The residue was taken up in a small volume of methanol and applied to Toyo-Roshi No. 51 paper.

As shown in Table IV, the R_f values of the hydroxamate isolated are consistent with those of authentic formohydroxamate in five different solvent systems.

TABLE IV

Identification of the Reaction Product in Formate Activation in the Presence of Hydroxylamine

Chromatographic solvent systems	R_f values	
	Authentic formohydroxamate	Reaction product
1. Butanol saturated with water	0.42	0.43
2. *tert*-Amyl alcohol saturated with water	0.60	0.61
3. Butanol : formic acid (85 per cent) : water (4 : 1 : 1)	0.46	0.46
4. Butanol : ammonia (28 per cent) : water (5 : 1 : 1)	0.04	0.04
5. Butanol : methyl ethyl ketone : ammonia (28 per cent) : water (5 : 3 : 1 : 1)	0.11	0.11

To find the degradation product of ATP during formate activation, similar experiments to those described above were performed either in the presence or absence of hydroxylamine. After incubation, the reaction mixture was deproteinized with perchloric acid. Nucleotides in the supernatant were adsorbed on charcoal and eluted with 50% ethanol containing 0.3 *N* ammonia (*15*). An aliquot of the eluate was applied to Toyo-Roshi No. 51 paper and developed with the solvent system used by Magasanik *et al.* (*13*).

Paper chromatograms from this experiment revealed that ADP is the major product formed from ATP during the formokinase reaction either in the presence or absence of hydroxylamine.

Stoichiometry—Qualitative paper chromatography showed that formohydroxamic acid and ADP accumulated during formate activation. The reaction mechanism of formokinase was studied further by stoichiometric analysis of the reaction.

For this purpose, an incubation was carried out as described in Table V. After incubation, a 3 ml. aliquot of the reaction mixture was used for the determination of formohydroxamate.

Another 3 ml. aliquot was deproteinized by adding trichloroacetic acid to a final concentration of 5 per cent. After removal of the precipitate by centrifugation, the supernatant was treated twice with 100 mg. of acid-treated charcoal to remove nucleotides. The charcoal fractions were combined and washed once with 5 ml. of 5% trichloroacetic acid. The supernatant from the charcoal treatment was combined with the washings and adjusted to 10 ml. with 5% trichloroacetic acid. One milliliter aliquots were used for the determination of inorganic ortho- and pyro-phosphate. The charcoal fraction was treated twice with 5 ml. of 50% ethanol containing 0.3 *N* ammonia and the combined eluates were analyzed for nucleotides.

Table V summarizes typical results of these analyses. The data clearly demonstrate the liberation of ADP and inorganic orthophosphate in stoichiometric accord with the formation of formohydroxamate. On the other hand, a formate-dependent formation of AMP and inorganic pyrophosphate was negligible. Therefore it is obvious that ATP splits to ADP and inorganic orthophosphate during formate activation in the presence of hydroxylamine.

Reversibility of the Formokinase Reaction—When the incubation was carried out in the absence of hydroxylamine, an unknown compound accumulated concomitantly with ADP. This reacted with hydroxylamine, forming a hydroxamate which was identified as formohydroxamate by paper chromatography. Furthermore this unknown compound was not adsorbed on charcoal and was labile at an alkaline pH. From these facts, it is concluded that the actual intermediate in formate activation is formyl phosphate rather than formyl adenylate.

If formyl phosphate is the true intermediate, it is possible that formokinase could also catalyze the backward reaction; that is, the formation of ATP from ADP and synthetic formyl phosphate. Table VI shows that this is the case. Table VI also indicates that the backward reaction like the forward reaction requires manganese as a cofactor. It must

TABLE V

Stoichiometry of Formokinase Reaction in the Presence of Hydroxylamine

	Complete system	Complete system minus formate	Difference
	μmoles	μmoles	μmoles
Formohydroxamate formed	3.86	0	3.86
Orthophosphate liberated	5.68	1.52	4.16
Pyrophosphate liberated	0.03	0.00	0.03
ADP formed	5.93	2.30	3.63

The complete system contained; 1.2 mmole of potassium formate, 20 μmoles of ATP, 40 μmoles of $MnCl_2$, 12 μmoles of 2-mercaptoethanol, 4 mmoles of hydroxylamine (neutralized with KOH), 300 μmoles of Tris buffer, pH 7.2, 250 μg. of formokinase (Step V), and water to a final volume of 6 ml. Incubation was carried out at 37°C for 30 minutes.

TABLE VI

ATP Formation from Formyl Phosphate and ADP

Experiment No.	Metals	ATP formed
		μmole
1	$MnCl_2$	0.59
2	$MnCl_2$	0.51
3	$MgCl_2$	<0.1

The reaction system contained 5 μmoles of formyl phosphate, 10 μmoles of ADP, 4 μmoles of 2-mercaptoethanol, 20 μmoles of $MnCl_2$ or $MgCl_2$, 100 μmoles of Tris buffer, pH 7.0, 125 μg. of formokinase (Step V), and water to a final volume of 2 ml. Incubations were carried out at 37°C for 20 minutes. After incubation, the reaction was stopped by adding 0.5 ml. of 25% perchloric acid. After centrifugation and neutralization with KOH, the supernatant was adjusted to 3 ml. with water and the ATP measured. Residual formyl phosphate was almost completely destroyed by this treatment.

be mentioned, however, that the values in the table are unsatisfactory for calculation of the equilibrium constant of the reaction because of the instability of the formyl phosphate.

Substrate Specificity—From the preceding experiments, it was observed that the characteristics of formokinase are quite similar in several respects to those of acetokinase reported by Rose *et al.* (16). Therefore it is of importance to know whether formokinase is identical with acetokinase or not. In the present report, nothing definite can be said about substrate specificity of the formokinase, because our preparation is still contaminated with acetokinase. However several results strongly suggest a difference between these two enzymes.

1) During purification of the enzyme, the activity ratio of acetokinase to formokinase decreases gradually, for example it was 4.1 in the crude extract and 1.5 at Step V (Table VII).

2) The distribution of the activities during ammonium sulfate fractionation are different. The highest formokinase activity is found in the fraction precipitating between 0.53 and 0.58 saturation, while that of acetokinase is obtained in the fraction precipitating between 0.58 and 0.65 saturation (Fig. 4). When the 0.53–0.58 fraction is further precipitated with acetone (50–55%), the specific activity of formokinase usually rises about 3-fold, whereas that of acetokinase does not (Fig. 4).

TABLE VII

Comparison of Activities of Formokinase and Acetokinase during Enzyme Purification

Preparation	Specific activity[1]		Acetokinase / Formokinase
	Formo-kinase	Aceto-kinase	
Crude extract	1.8	7.4	4.1
1st Acetone fractionation	4.2	15.9	3.8
Calcium phosphate gel treatment	5.2	19.3	3.8
Ammonium sulfate fractionation	28.5	83.5	2.5
2nd Acetone fractionation	82.0	121.0	1.5

1) Units per mg. of protein.

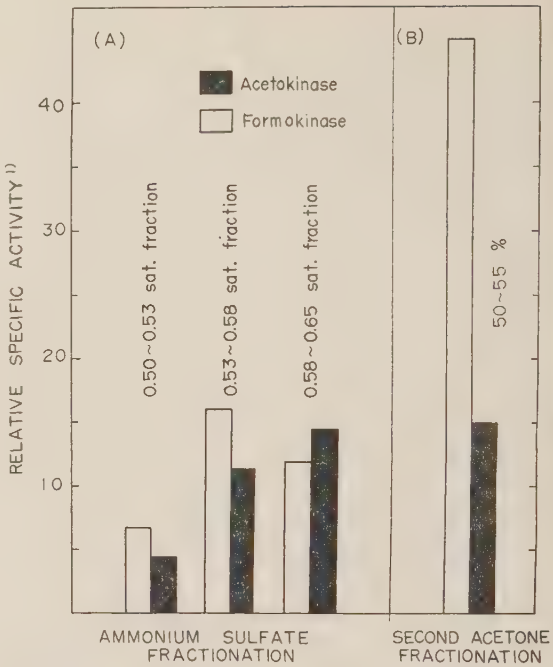

FIG. 4. Distribution of formokinase and acetokinase during ammonium sulfate and acetone fractionation. Step III enzyme was fractionated with ammonium sulfate (A). 0.53~0.58 saturated ammonium sulfate fraction was further subjected to acetone fractionation (B). For assay conditions see text.

1) Relative specific activity on ordinate expresses the ratio of the specific activities of both enzymes at each fraction indicated to those of the crude extracts.

TABLE VIII

Comparison of Metal Requirements of Formokinase and Acetokinase

Enzyme	Metal added	Hydroxamate formed	
		With formate[1]	With acetate[2]
Crude extract	Mn^{++}	2.09	2.24
	Mg^{++}	0.73	2.51
Step V enzyme	Mn^{++}	1.69	3.45
	Mg^{++}	0.46	3.73

2.71 mg. of crude extract protein were used with formate and 67.7 μg. with acetate, and 62 μg. of Step V enzyme with formate and 6.2 μg. with acetate.

1) Micromoles per 20 minutes.

2) Micromoles per 5 minutes.

3) The metal requirements of these two enzymes differ. Acetokinase is activated to the same degree by either manganese or magnesium, while formokinase is stimulated far more by manganese than magnesium (Table VIII and Fig. 3).

These facts strongly suggest that formokinase differs from acetokinase.

Table IX

Effect of SH-inhibitors on Formate Activation

Experiment No.	Inhibitor	Concentration of inhibitor	Concentration of $MnCl_2$	L-Cysteine	Hydroxamate1) formed	Inhibition
		$\times 10^{-3}M$	$\times 10^{-3}M$	$\times 10^{-2}M$		per cent
1	None		3.33		1.20	
	p-Chloromercuribenzoate	0.2	3.33		0.05	96
		0.2	3.33	1.0	1.25	0
2	None		3.33		0.89	
	$HgCl_2$	2.0	3.33		0.68	24
	None		0.67		0.73	
	$HgCl_2$	2.0	0.67		0.17	86
	$HgCl_2$	2.0	0.67	1.0	0.73	0

In Experiment 1, the reaction mixture contained: 15 μmoles of ATP, 2 mmoles of hydroxylamine (neutralized with KOH), 10 μmoles of $MnCl_2$, 150 μmoles of Tris buffer, pH 7.2, 0.6 μmole of *p*-chloromercuribenzoate, crude extract (4.84 mg. of protein) and water to a total volume of 2.4 ml. After 5 minutes preincubation at 37°C, 0.5 ml. of formate (600 μmoles) and 0.1 ml. of cysteine or water were added and the incubation was continued for another 20 minutes at 37°C.

In Experiment 2, the system consisted of 600 μmoles of formate, 15 μmoles of ATP, 2 mmoles of hydroxylamine, 150 μmoles of Tris buffer, pH 7.2, $HgCl_2$, crude extract (4.84 mg. of protein) and water to a total volume of 2.8 ml. After 5 minutes incubation at 37°C, 0.1 ml. each of $MnCl_2$ and of cysteine or water were added and the incubation was continued for another 20 minutes at 37°C.

1) Micromoles per 20 minutes.

Inhibitors—Even in a crude extract which does not require SH-compounds for maximal activity, formate activation is strongly inhibited by SH-blocking agents such as *p*-chloromercuribenzoate and heavy metals. The inhibition is overcome by the addition of SH-reagents (Table IX). It must be noted that the degree of inhibition by heavy metals depends on the concentration of manganese in the medium, *viz*., the inhibition increases in inverse proportion to manganese concentration (Table IX, Experiment 2). The data suggest that manganese competes with heavy metals for the sulfhydryl group of the enzyme. Formokinase is also completely inhibited by EDTA at a final concentration of $1\times 10^{-2}M$. No appreciable inhibition is produced by 0.05 M fluoride.

Other Properties—The half saturation concentration (*Km* value) of formate is 0.60 M which is in the same order as that of acetate in the acetokinase reaction. This shows that the affinity of formokinase for formate is quite low.

The enzyme activity is the same in aerobic and anaerobic conditions.

Heat treatment at 50°C for 5 minutes causes a decrease in the enzyme activity of 40 per cent, at 60°C for 5 minutes of 70 per cent.

Formokinase is also found in extracts of *Pseudomonas fluorescens* A_{3-12}, *Clostridium welchii* and *Aerobacter aerogenes*.

DISCUSSION

It is clear from the present report that formokinase catalyzes the following reaction,

$$\text{Formate}+\text{ATP}\rightleftharpoons\text{formyl phosphate}+\text{ADP} \qquad (1)$$

and, if hydroxylamine is present:

$$\text{Formyl phosphate}+\text{hydroxylamine}\xrightarrow{\text{nonenzymatic}}\text{formohydroxamate}+i\text{P} \qquad (2)$$

$$\text{Net (1)+(2): formate}+\text{ATP}+\text{hydroxylamine}\longrightarrow\text{formohydroxamate}+\text{ADP}+i\text{P} \qquad (3)$$

The reaction (reaction 1) is quite similar to that of acetokinase which forms acetyl phosphate from acetate and ATP. Therefore it is evident that the formokinase reaction belongs to the Type 2 category of activation of Lipmann (*17*).

The physiological significance of formokinase is not clear at present. According to Greenberg and Jaenicke (*18*), the formyl group of chemically-synthesized formyl phosphate is not transferred to THFA by a pigeon liver enzyme. However, judging from our studies, their method of synthesis of formyl phosphate is questionable. Therefore the transfer reaction between formyl phosphate and THFA must be reinvestigated with a well-identified preparation of formyl phosphate.

By analogy with acetate activation, another possible role of formokinase is concerned with condensation reactions perhaps with the participation of CoA. Studies on these lines are now under way.

SUMMARY

1. A new formate activating enzyme, formokinase, has been purified approximately 60-fold from extracts of acetone-dried cells of *E. coli*, strain B, by means of acetone precipitation, treatment with calcium phosphate gel, and ammonium sulfate fractionation.

2. Formokinase catalyzes the formation of formohydroxamate in the presence of formate, ATP and hydroxylamine. Stoichiometric formation of formohydroxamate, ADP, and inorganic orthophosphate were demonstrated.

3. The reaction requires a sulfhydryl compound (glutathione, cysteine, thioglycolate, or 2-mercaptoethanol) and manganese ion. The participation of folic acid derivatives and CoA in the reaction seems unlikely.

4. Formokinase also catalyzes the formation of ATP from synthetic formyl phosphate and ADP in the presence of manganese ion. Therefore it is concluded that formyl phosphate is the actual intermediate in the formokinase reaction.

5. SH-blocking agents such as *p*-chloromercuribenzoate or heavy metals and EDTA are potent inhibitors of the enzyme.

6. Formokinase is also found in extracts of *Pseudomonas fluorescens* A_{3-12}, *Clostridium welchii* and *Aerobacter aerogenes*.

7. Some evidence differentiating formokinase from acetokinase is presented.

The authors wish to express their thanks to Dr. M. Suda for the benefit of many fruitful discussions. The authors also wish to express their gratitude to Dr. R. Y. Stanier, University of California, U.S.A., for a gift of *Pseudomonas fluorescens* A_{3-12} and to Dr. M. Yamanaka, Osaka Medical College, for a gift of *Clostridium welchii*.

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Studies on Hemoglobin

V. Chromatographic Resolution of Bovine Globin into the Valylleucyl and Methionyl Chains

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(Received for publication, August 27, 1960)

Bovine hemoglobin possesses two pairs of N-terminals, valylleucyl (α) and methionyl (β), per 6.8×10^4 g. (*1*, *2*) which has been presumed to be the most probable molecular weight (*3*). The evidences for the presence of hemoglobin as a dimer of same (or similar) subunits were also derived from the crystallographic analysis with X-ray (*4*), from the change of the molecular weight under the action of urea (*3*) or diluted acid (*3*, *5*) and from the analysis on the thiol groups (*6*, *7*) and on the peptides obtained by the proteolysis (*8*, *9*, *10*).

On the resolution of the two different chains of hemoglobin, on the other hand, there have been no reports until recent time. In 1959, Wilson and Smith (*11*) succeeded in separating the valylleucyl and valylglutamyl chains of horse globin* by the elution chromatography, and a little later, Ingram (*13*) proved the method to be also effective for the fractionation of α and β chains of human globin. Recently Take (*14*) found that the more complete and reproducible resolution of hemoglobin subunits could be achieved by the continuous electrophoresis on a paper curtain impregnated with concentrated urea.

The present paper describes that the two different chains, valylleucyl (α) and methionyl (β) chains of bovine hemoglobin were able to be separately eluted from a column of Amberlite CG-50 of H-phase with urea in 2 *N* formic acid, stepwise increasing the former concentration from 4, 6 to 8 *M*.

EXPERIMENTAL

Meterials—Bovine hemoglobin and globin were prepared according to the method as already reported by Satake (*1*, *8*).

Oxidized globin was obtained by the performic acid-oxidation of globin, according to the method of Sanger (*15*) who oxidized the disulfide linkages of insulin.

Amberlite CG-50, type I (150–200 mesh) used for the following chromatography, was purified by the successive washing with *N* hydrochloric acid, water, acetone, water, *N* sodium hydroxide, water, *N* hydrochloric acid, and water, according to the method of Hirs *et al.* (*16*).

Chromatography—Two hundred mg. of bovine hemoglobin, globin, or the oxidized preparation was dissolved into 10 ml. of 2 *N* formic acid containing urea at a concentration of 8 *M*. The viscous solution, after standing at room temperature for 2 to 3 days, was added to a suspension of 5 g. of Amberlite CG-50 (H-phase, dry weight) in 20 ml. of 2 *N* formic acid, under a vigorous stirring. The resulting suspension, in which the most part of protein was adsorbed on the resin, was directly added to a column of Amberlite CG-50 (H-phase) of 1×20 cm., previously equilibrated with 2 *N* formic acid. After the addition of resin suspension which had adsorbed globin, the height of the resin layer increased to 28 cm.

As soon as the supernatant of the applied suspension had been allowed to flow into the column, the protein adsorbed on the column was washed with 75 ml. of 2 *N* formic acid, and then eluted with urea in 2 *N* formic acid. The urea concentration was increased stepwise from 4, 6 up to 8 *M*. The rate flow was maintained to be 5 ml. per cm^2 per hour, throughout these procedures.

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* Horse globin, differing from bovine one, has three different polypeptide chains (*1*, *2*), which have been separated from each other by Take and by Satake *et al.* (unpublished) (see also (*11*, *12*)).

Analytical Methods—The protein concentration in the chromatographic effluent fractions which had been collected in 5 ml.-fraction by means of an automatic fraction collector, was determined by direct spectrophotometry at 280 mμ and by the turbidity produced in *M* trichloroacetic acid,* as described by Satake *et al.* (*17*).

The combined effluent fractions corresponding to each peak part (see Figs. 2 and 4), were dialyzed against running tap water with the use of cellulose casing (Visking Company, Division of Union Carbide Corporation). After 2 to 3 days, all the contents of the dialysis sac (precipitates usually being in evidence) were dried in vacuum from the frozen state. Preliminary experiments indicated that the recoveries of protein after dialysis, were almost quantitative.

Paper electrophoresis was performed on Toyo Roshi No. 51 filter paper of 5×30 cm., with the use of a mixture of acetic acid (1 ml.), pyridine (30 ml.), and urea (360 g) in one liter of water, pH 6.8, as the background electrolyte (*14*). Bromophenol blue reagent was used for the detection of protein on the paper strip.

The N-terminals of the protein were analyzed with the usual 1-fluoro-2, 4-dinitrobenzene method of Sanger. Hydrolysis of DNP-protein was performed with 6 *N* hydrochloric acid at 110°C for 5 hours. Ether-soluble DNP-amino acids and -peptide derived from the *N*-terminals, were identified by their chromatograpaic *Rf* (see Fig. 1).

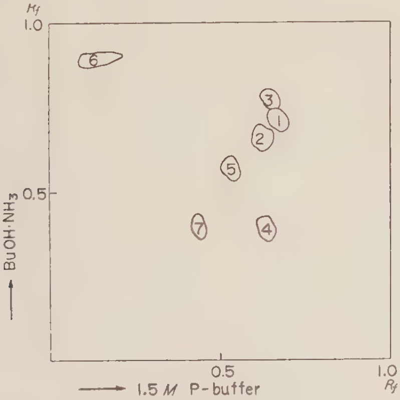

FIG. 1. Paper chromatogram of DNP-compounds derived from DNP-globin.

1. DNP-Valine
2. DNP-Methionine
3. DNP-Valylleucine
4. DNP-Methionine sulfone
5. 2, 4-Dinitrophenol
6. 2, 4-Dinitroaniline
7. ε-mono-DNP-Lysine

RESULTS

Fig. 2 shows the elution diagram of bovine globin which has been previously treated at room temperature for 2 days with 8 *M* urea in 2 *N* formic acid. The pre-incubated globin solution was added to the column, after the dilution with a suspension of Amberlite CG-50 in 2 *N* formic acid (the concentration of urea being decreased from 8 *M* up to 3 *M* and most part of the protein being adsorbed on the resin), and the elution was achieved with urea, increasing the concentration from 4, 6 to 8 *M*. Two protein fractions were separately eluted with 6 *M* and 8 *M* urea, and the total recoveries from the column were between 80 and 90 per cent. Under the same conditions, hemoglobin previously treated with acidic urea, was also resolved into two protein fractions, and their elution positions and amounts (as determined by trichloroacetic acid-turbidimetric method) were essentially the same as those derived from globin, respectively (40–45 per cent with 6 *M* urea and 45–50 per cent with 8 *M* urea).

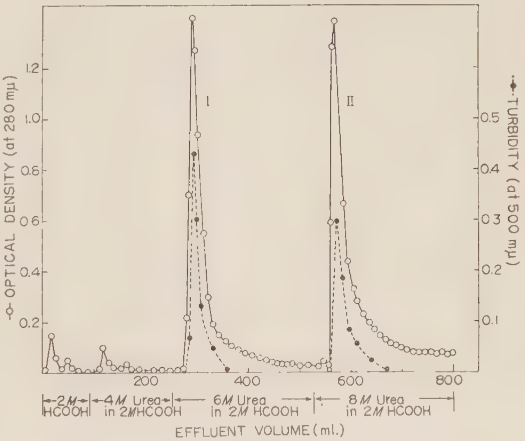

FIG. 2. Chromatographic resolution of bovine globin.

Column—Amberlite CG-50 of H-phase, 1×28 cm.

Effluents—4, 6 and 8 *M* urea in 2 *N* formic acid

—○—, indicates protein concentration as determined by ultraviolet spectrophotometry.

—●—, indicates protein concentration as determined by turbidimetric method.

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* The readings are expressed as an optical density at 500 mμ of an equi-volume mixture of five times-diluted sample solution and 2 *M* trichloroacetic acid.

TABLE I

Chromatography of Bovine Globin under Various Conditions

No.	Conditions of Chromatography	Amounts of the Fractions eluted with	
		6*M* urea	8*M* urea
1	Optimum Conditions1)	40, 45, 43% (α)	45, 47, 50% (β)
2	Pre-incubation 5 hrs., 20°C2) 10 hrs., 20°C 20 hrs., 20°C 30 min., 80°C	17% (α) 29% (α) 39% (α) 42% (α)	78% ($\beta+\alpha$) 65% ($\beta+\alpha$) 57% ($\beta+\alpha$) 48% (β)
3	Pre-incubated sample was directly added to column2)	Some amounts (15–65%) passed through the column without adsorption	
4	Eluted with urea at pH 1.42) (HCl instead of formic acid)	α and β were eluted with 4–5*M* urea without complete resolution	
5	Eluted with urea at pH 2.52) (acetic acid instead of formic acid)	broad and low peak a %3)	79% ($\alpha+\beta$)
6	Pre-incubated with 8*M* urea, pH 7 and eluted with urea, pH 7, from a column of Na-phase	α and β were scarcely adsorbed	

1) See Fig. 2.
2) Other conditions were just the same as the optimum ones.
3) a was impossible to be estimated.

TABLE II

N-Terminals of Bovine Globin and the Subunits

Globin	N-Terminals (as DNP-derivative)1)				
	Valine	Valylleucine	Methionine	Methionine Sulfone	Others
Whole globin	⧻	⧺	⧻	(+)2)	—3)
Pre-incubated with acid urea	⧻	⧺	⧻	(+)	—
Oxidized with performic acid	⧻	⧺	—	⧻	—
Globin fraction eluted with 6*M* urea with 8*M* urea	⧻ trace	⧺ —	— ⧻	— (+)	— —
Oxidized globin eluted with 6*M* urea with 8*M* urea	⧻ trace	⧺ —	— —	— ⧻	— —

1) DNP-Protein was hydrolyzed with 6*N* HCl at 110°C for 5 hours.
2) Detected only with the use of commercial ether (without purification) for the extraction.
3) — indicates the amounts less than 0.1 mole per 10^5 g.

Fig. 3 summarizes the paper electrophoretic patterns of bovine globin and of the fractions resolved chromatographically. The results clearly indicated that the protein components eluted with $6\,M$ urea and with $8\,M$ urea, possessed electromobilities different from each other. Such a difference may be in reference to their chemical composition or to their molecular size, due to the dissociation of globin under the action of concentrated urea (*11*).

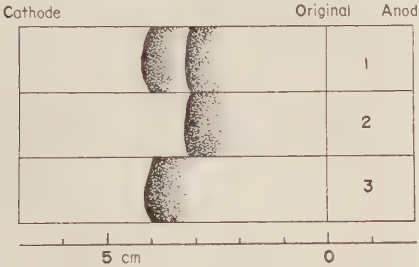

FIG. 3. Paper electrophoretic patterns of bovine globin and the subunits.
Sample—1. Whole globin
2. Globin eluted with $8\,M$ urea
3. Globin eluted with $6\,M$ urea
Electrophoresis was performed under a potential gradient of 20 volt per cm., for 2 hour. Six molar urea in acetic acid-pyridine-water, pH 6.8, was used as the background electrolyte.

Fig. 4 represents the elution diagram of oxidized bovine globin. All the procedures for the pre-incubation of the protein sample, for the adsorption of the sample on the resin and for the elution chromatography, were just the same as mentioned above. Two components were separately eluted at the concentration (as determined by turbidimetric method) similar to those of the corresponding fractions derived from unoxidized globin, respectively. There was observed, however, much less ultraviolet absorption in both of the oxidized globin fractions, as compared with those of the corresponding unoxidized ones. These facts seemed to indicate that the both components possessed tryptophan and tyrosine residues.*

The conditions described above rather in detail were important for the successful resolution of globin. Some results obtained under other conditions, are summarized in Table I.

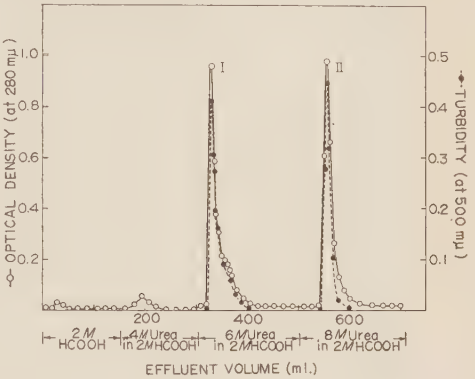

FIG. 4. Elution diagram of oxidized bovine globin.

Conditions and methods used were just the same as shown in Fig. 2.

The N-terminal analysis of the both components further verified that their difference in the elution chromatography and in the paper electrophoresis, was in reference to their amino acids composition. Thus the globin eluted with $6\,M$ urea (faster moving component to cathode), possessed valylleucyl, while that eluted with $8\,M$ urea (slower moving component to cathode) had methionyl as their N-terminal, respectively. These results are summarized in Table II.

DISCUSSION

From the above-mentioned results, it was elucidated that the valylleucyl (α) and menthionyl (β) chrins of bovine globin could be fractionally eluted from a column of Amberlite CG-50 of H-phase with the use of urea in $2\,N$ formic acid, as the effluent. Recently, Take (*14*) also succeeded in the electrophoretic resolution of bovine hemoglobin into two protein fractions. By the comparison of the paper electrophoretic diagrams of these protein fractions (Fig. 3), and of their tryptic hydrolysates (*14*), it was confirmed by Take and by the present author that the valylleucyl (α) and methionyl (β) chains obtained by the present author corresponded to the electrophoretically faster and slower moving components by Take, respectively.

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* Tryptophan residue, differing from tyrosine residue, changed to a residue having much less ultraviolet absorption by the performic acid-oxidation.

By the densitometry of the protein fractions on the paper electrophoretic pattern, the relative amounts of the faster (α) and slower (β) moving components were estimated to be approximately 45-50 and 50-55 per cent in weight, respectively (*14*). The values were not so inconsistent with their amounts resolved by the elution chromatography (α: 40-45 per cent and β: 45-50 per cent).

Important for the chromatographic resolution of globin were the conditions (Table I). In general the lower the pH of effluent, the less the adsorption of both subunits on Amberlite CG-50 of H-phase. At low pH, both components were rapidly eluted without any resolution (Table I, No. 4), while at high pH, each component was eluted only with a tremendous tailing (Table I, No. 5). Ambelite CG-50 of Na-phase, however, did not adsorbed globin at all (Table I, No. 6). Formic acid was used, instead of hydrochloric acid (*11*, *15*) for practical purpose to maintain the pH in an optimum range, which was rather narrow.

Pre-incubation of globin with acidic urea was essential for the reproducible resolution. It was because that globin added as 2 *N* formic acid solutin, was scarcely eluted with 6 *M* urea, and all proteins were eluted as a single peak with 8 *M* urea. The period during which globin was treated with 8 *M* urea in 2 *N* formic acid, showed also a remarkable effect on the resolution. The amount of protein eluted with 6 *M* urea (α), increased from 0-5 per cent up to 40-45 per cent with increment of the pre-incubation period from 0 to 1-3 days (Table I, No. 1, No. 2). The period for the optimum resolution could be greatly shortened by elevating the temperature of pre-incubation (for example, 30 minutes as 80°C; see No. 2 in Table I).

Globin solution in 8 *M* urea so incubated, was scarcely adsorbed on Amberlite CG-50 of H-phase, unless the concentration of urea was lowered up to 4 *M*; while the dilution of urea concetration was accompanied with a rapid irreversible change of globin subunits into an aggregate (*11*), which was eluted only with difficulties.

The presence of Amberlite CG-50 during the dilution, seemed to inhibit such an aggregation, presumably due to the adsorption of the both components from the solution.

SUMMARY

1. Bovine hemoglobin or globin was resolved into two different subunits, valylleucyl (α) and methionyl (β) chains, by the acidic urea-treatment followed by the elution chromatography from a column of Amberlite CG-50 of H-phase with the use of urea in 2 *N* formic acid, increasing the former concentration stepwise from 4, 6 to 8 *M*.

2. Under the similar conditions, performic acid-treated globin was also resolved into the both oxidized chains.

The auther is greatly indebted to Mr. Take, Showa College of Pharmacy, for advancing information pertinent to his own experimental data on bovine hemoglobin. Her gratitude is also expressed to Prof. K. Satake for his interest and discussion, and to Mr. Y. Kimura, Miss M. Yoshida and Miss Y. Nagatsuka for their assistance in analysis of the column eluates.

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On the Dissociation of Hemoglobin under the Action of Urea

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A number of studies has demonstrated that the molecular weight of mammalian hemoglobin is 6–7×10^4 (*1*, *2*). Under the action of urea or dilute acid, however, the molecular weight is about a half of this, indicating that the protein is able to dissociate into two halves (*1*). Further evidences for the presence of hemoglobin as a dimer of same (or similar) subunits, are derived from the *N*-terminal content (*3*, *4*, *5*,), from the sulfhydryl group analysis (*6*), from the arginine-peptide composition (*7*, *8*), and from the 'finger print' diagram of the tryptic hydrolysate (*9*).

Hemoglobins from various vertebrates, however, are mostly composed of four hemes and pairs of two different polypeptide chains; thus bovine hemoglobin possesses two valylleucyl (α) and two methionyl (β) chains per 6.8×10^4 g. (*3*, *4*). The fact seems to indicate that the protein may dissociate into two halves either in symmetrical or in asymmetrical way.

$$\begin{tikzcd} & 2\alpha\beta \text{ (symmetrical dissociation)} \\ \underset{\text{Hemoglobin}}{\alpha_2\beta_2} \arrow[ur] \arrow[dr] & \\ & \alpha_2+\beta_2 \text{ (asymmetrical disociation)} \end{tikzcd}$$

Recently the dissociation of human hemoglobin was proved to be asymmetrical one, from the studies of Vinograd who analyzed the C^{14}-contents of normal and of sickle cell anemia hemoglobins reconstructed from a mixture of labeled HbA and unlabeled HbS, which had been previously dissociated into two halves under the action of dilute acid (*10*).

In this paper it will be described that the urea-dissociation of bovine hemoglobin in neutral solution, seemed also to preceed asymmetrically and that the valylleucyl (α) and the methionyl (β) chains so dissociated, could be esily resolved by the paper electrophoresis in the presence of 6 *M* urea.

EXPERIMENTALS

Materials—Boving hemoglobin and globin used were the same preparations as used by S. Sasakawa (*7*, *11*).

Trypsin used for the partial hydrolysis of globin subunits, was a twice crystrallized and salt-free preparation from Worthington Biochemical Corporation.

Filter paper used for electrophoresis, was Toyo Roshi No. 50.

Paper Electrophoresis—Analytical electrophoresis of hemoglobin, whole globin, or globin subunits was performed on a paper strip of 5×30 cm. at a constant potential gradient of 14 volts per cm. (Paper Electrophoresis Apparatus Model II-B, Toyo Roshi Co. Ltd.).

As the background electrolyte, was used 6 *M* urea in *M*/25 acetate- *M*/50 veronal buffer (pH 4–8) in *M*/20 carbonate-bicarbonate buffer (pH 8.5–10), or in a mixture of pyridine (3–30 ml.), acetic acid (3–30 ml.) and water (total volume 1000 ml., pH 4–6.5). After the two hours-electrophoresis, the paper strip was dried at 80°C, and treated with bromophenol blue reagent (*12*), to stain the position of protein. By the densitometry of the color at 570 mμ, the amount of protein was estimated. As the standard of this assay, the corresponding protein component isolated according to the following methods, was used.

Continuous electrophoresis in preparative scale, was carried out with the use of Grassmann-type apparatus (*13*) (Mitamura Rikagaku Kikai Co. Ltd.) on a paper curtain of 50×45 cm. which possessed 48 serrate tubs for the drip point (one tube per cm., see Fig. 3). Under a constant electric current of 20 mA (at 450–500 volts), sample solution in 6 *M* urea at a concentration of 10 mg. per ml., was applied on the paper curtain at a flow-rate of 0.1 ml. per hour, while the background electrolyte was allowed to flow at a rate of 0.1–0.2 ml. per hour per cm. of the cross-section (per one dripping tube).

Analysis of Globin Subunits—An aliquot of protein fraction resolved by the above-mentioned preparative electrophoresis, was directly applied on paper strip (as 6 *M* urea solution) for the analytical re-electrophoresis, while the remaining main part was separately dialyzed and lyophilized. Preliminary experiments showed that the protein scarcely escaped through the dialysis membrane (cellulose casing, Visking corporation).

The peptide constituents derived from each lyophilisate with trypsin, were analysed by the paper electrophoresis, according to the method of Sasakawa.* Thus the tryptic hydrolysis was performed in *M*/10 borate buffer at pH 8.5 and at 35°C, with a ratio of enzyme/substrate of 1/50 (w/w) for 6 hours. Paper electrophoresis was performed at a constant potential gradient of 20 volt per cm., using a mixture of pyridine (30 ml.), acetic acid (4 ml.) and water (965 ml.) (pH 6.5) as the background electrolyte. After the three hours-electrophoresis, the paper strip was treated with ninhydrin-, Millon-, Ehrlich-, and Sakaguchi-reagents to stain the position of peptides.

RESULTS

Fig. 1 A, B, and C show the paper electrophoretic patterns of bovine hemoglobin and globin at pH 6.8 in the absence or presence of 6 *M* urea respectively. The results seemed to indicate that hemoglobin or globin in 6 *M* urea solution presented as a mixture of two protein components different from each other in their electrophoretic mobility.

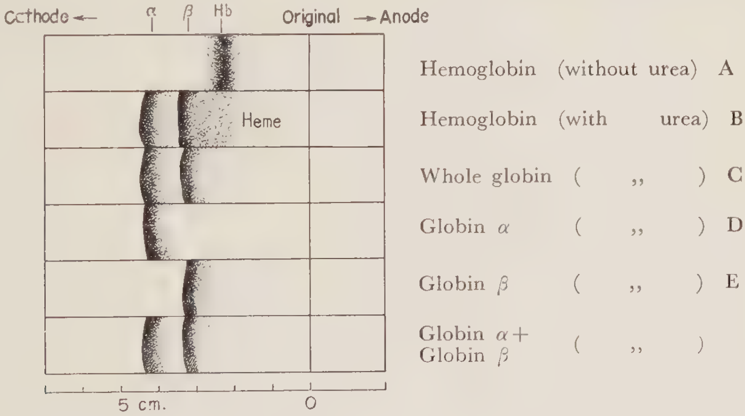

FIG. 1. Paper electrophoretic patterns of whole globin and the subunits. Background electrolyte: 6 (or 0) *M* urea in a mixture of 4 ml. acid, 30 ml. pyridine, and 966 ml. water (pH 6.8). The distance shown, was not corrected from the electro-osmotic flow.

Such an electrophoretic resolution of globin into two components under the action of urea, was observed in a range of pH higher than 5.5, either the sample solution was applied on paper strip as hemoglobin or as globin. In the absence of sufficient concentration of urea (approximating more than 4 *M*) in the background electrolyte, on the other hand, there was no indication of the resolution, even though hemoglobin or globin had been previously treated with concentrated urea (*11*) and applied as the 6 *M* urea solution. The electrophoretic mobilities of the both protein components in 6 *M* urea at various pH, are summarized in Fig. 2, together with those of intact hemoglobin in the absence of urea.

Fig. 3 illustrates the continuous electrophoretic diagram of bovine globin in 6 *M* urea solution at pH 6.8. The both components, faster and slower moving components to the cathode, were electrophoretically homogeneous (see Fig. 1 D and E) and did not interchange from one component to the other, as far as examined. The relative amounts of the faster and slower moving components, were estimated to be about 55 and 45 per cent in weight, respectively. These results might suggest that the difference between the both components would be in reference to their chemical composition rather than to their molecular size, due to a reversible dis-

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*Unpublished (see also *14*, *15*, *16*).

sociation or to a re-association under the action of urea. (*17*).

The presumption was partly verified from

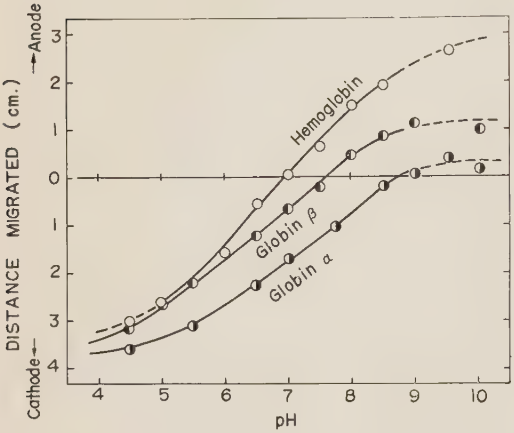

Fig. 2. Electrophoretic mobilities of hemoglobin and of globin subunits. Background electrolyte: 6 *M* urea in *M*/25 acetate- *M*/50 veronal buffer (pH 4–8) or in *M*/20 carbonate-bicarbonate buffer (pH 8.5–10). In the case of hemolobin, urea was omitted from the mixture. The electro-osmotic flow was corrected in all cases.

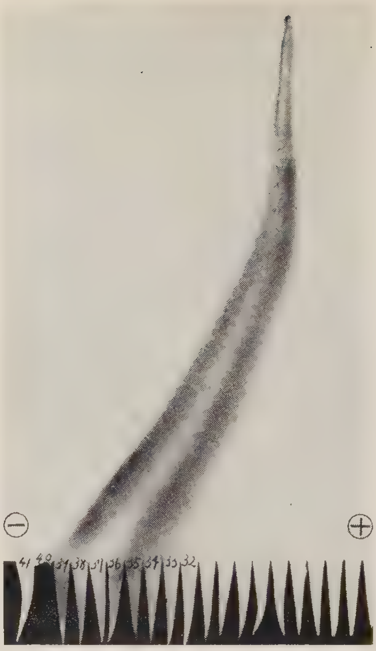

Fig. 3. Continuous electrophoresis of bovine globin on a paper curtain. A mixture of 30 ml. pyridine, 4 ml. acetic acid, 360 g. urea in one liter of water, was used as the background electrolyte.

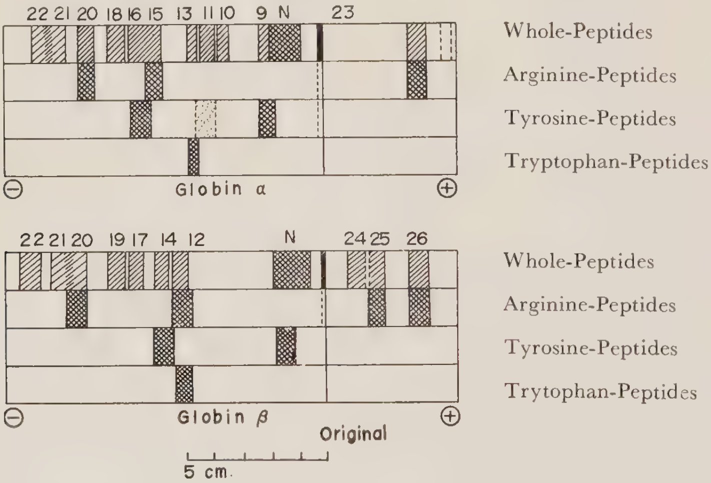

Fig. 4. Paper electrophoretic diagrams of petides derived from the faster (α) and from the slower (β) moving componets of bovine globin.

Sample: Tryptic hydrolysates of globin subunits, α and β.

Detection: Ninhydrin for whole peptides, Sakaguchi-reagent for arginine-peptides, Millon-reagent for tyrosine-peptides, and Ehrlich-reagent for tryptophan-peptides.*

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*The number indicates each peptide No., which was derived from human globin with trypsin and migrated to the position, under these conditions (*9*, *14*, *15*, *16*). N indicates a mixture of neutral peptides.

the peptide constituents of the both protein components. Fig. 4 presents the paper electrophoretic diagram of peptides derived from each component with trypsin. The results clearly indicated that the both components, faster and slower moving ones, possessed quite different arginine-, tyrosine- and tryptophan-peptides and differed throughout the whole structure, from the one to other.

DISCUSSION

Bovine hemoglobin or globin was resolved into two protein components, by the paper electrophoresis in the presence of 6 *M* urea (Figs. 1, 2 and 3). The both components so resolved, differed from each other in their chemical composition (Fig. 4). These facts seemed to suggest that hemoglobin molecule was associated from two different subunits by the non-covalent linkages, which were able to split under the action of urea.

Recently, Ingram (*17*) reported that the electrophoretic resolution of human hemoglobin into two components, was also possible under the action of dodecyl sulfate, too. As reported in the preceding paper of this Journal, Sasakawa (*11*) succeeded to separate two different chains of bovine globin by the elution chromatography with acidic urea as the eluent. By comparing the electrophoretic diagrams of the both components resolved by electrophoresis and by chromatography, it was kindly verified by Sasakawa (*11*), that the faster moving component obtained by the present author, coresponded to her valylleucyl chain (α), while the slower moving component to her methionyl chain (β), (see Fig. 3 in Sasakawa's report) (*11*), According to Sasakawa (*11*), bovine globin α (faster moving component) possessed one tryptophan, two tyrosine and three arginine residues, while β-chain (slow moving component) had one tryptophan, two tyrosine, and four arginine residues, respectively. The peptide constituents derived from the both components with trypsin (Fig. 4) will also verifed the above presumption.

The relative amounts of α and β chains estimated by the present author (45 per cent/55 per cent) were fairly consistent with the ratio of their minimum molecular weight ($1.5\times10^4/1.9\times10^4$) calculated by Sasakawa from the *N*-terminal content and the amino acid composition (*11*). As already mentioned, bovine hemoglobin possesses two valyllecyl (α) and two methionyl (β) chains per 6.8×10^4 g. (*3*, *4*), which has been presumed to be the most probable molecular weight (*1*, *2*). In this paper, were offered no evidences to decide whether the both components, α and β resolved by the electrophoresis, existed as the dimers (α_2, β_2) or as the monomers (2α, 2β) (*17*).

$$\begin{tikzcd} & \alpha_2+\beta_2 \arrow[dr] & \\ \alpha_2\beta_2\text{——or} \arrow[ur] \arrow[dr] & & 2\alpha+2\beta \\ & 2\alpha\beta \arrow[ur] & \end{tikzcd}$$

	hemoglobin	dimer	monomer
Mol. wt. $\times10^4$	6.8	3.5–4.0	1.5–1.9

Bovine hemoglobin in 6 *M* urea solution at neutral pH, however, has been reported to possess a molecular weight of 3.5 to 4.0×10^4 (*1*), thus approximating to a half of 6.8×10^4, and the both components have minimum molecular weights of rather similar magnitude. These facts seemed to suggest that the both components in 6 *M* urea solution present as their dimer, thus hemoglobin dissociates into two halves asymmetrically under the action of urea.

On the acidic acetone-treatment, hemoglobin dissociated into heme and globin, the molecular weight of which has been reported to be $3.5\text{–}4.0\times10^4$ (*1*, *2*), suggesting the dissociation under the action of acid (*10*).

In the present case, too, heme group of urea-dissociated hemoglobin was easily separated from the globin subunits, during the electrophresis. These facts will be derived from the lability of the linkages between the heme and the dissociated subunits.

SUMMARY

1. Bovine hemoglobin or globin was resolved into two different subunits by paper electrophoresis in 6 *M* urea at pH 6.5.

2. The components moving faster and slower to the cathode, corresponded to valyl-

lecyl (α) and methionyl (β) chains, respectively.

3. The both chains seemed to differ from each other not only at their N-terminal position but throughout the whole structure, from the electrophoretic patterns of their tryptic hydrolysates.

4. The possibility of the asymmetric dissociation of hemoglobin ($\alpha_2\beta_2 \longrightarrow \alpha_2+\beta_2$) under the action of urea, was discussed.

The author is greatly indebted to Mrs. S. Sasakawa, of Prof. Satake's Laboratory, Tokyo Metropolitan University, for advance information pertinent to her own experimental data on bovine hemoglobin. The author's gratitude is also expressed to Prof. K. Satake for his interest and discussion.

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The Journal of Biochemistry, Vol. 49, No. 3, 1961

The Synthesis of Stero-Bile Acids

XXXV. Formation of Bile Acids from Cholesterol in the Organism of *Rana catesbiana*

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(Received for publication, September 6, 1960)

In the bile of *Rana catesbiana* and *temporaria*, the frog family, a peculiar stero-bile acid, trihydroxycoprostanic acid is contained. This acid was discovered by Kazuno and Kurauchi (*1*) in 1939 and later the same acid was isolated from the bile of various members of the crocodilians by Haslewood (*2*, *3*).

It was shown that in mammals bile acids arise from cholesterol by oxidative cleavage of the terminal carbon atoms of its side chain and that trihydroxycoprostanic acid is converted to cholic acid in rats (*4*) and guinea pig (*5*). Trihydroxycoprostanic acid has since been regarded as an important intermediate in the conversion of cholesterol to cholic acid in animals. The assumption, however, awaits experimental evidence, and furthermore, metabolism of cholesterol in the bull frog is not known. In this report the conversion of cholesterol-4-C^{14} to trihydroxycoprostanic acid-4-C^{14} and cholic acid-4-C^{14} was verified in the organism of *Rana catesbiana*.

EXPERIMENTAL AND RESULTS

Three bull frogs weighing 250 g., to which 0.05 mc each of cholesterol-4-C^{14} in alcoholic suspension had been intraperitoneally injectted, were kept in a restraining cage for two weeks. The cholesterol-4-C^{14} was obtained from the Radiochemical Centre, Amersham, England. The bile obtained from three gall bladders was hydrolyzed with 2.5 N NaOH by heating in a sealed metal container for 8 hours at 160°C. The hydrolysate was poured into a large amount of water to separate bile sterol and then filtered. After acidifying the filtrate, bile acid was extracted with ether. The ether extract was washed with water to neutral and concentrated. The bile sterol and ether extract were then subjected to reversed phase partition chromatography by the use of the following solvent systems described by Bergström, Norman and Sjövall (*6–9*).

Phase system	Moving phase		Stationary phase	
A	Methanol	180	Chloroform	45
	Water	120	Heptane	5
C	Methanol	150	Chloroform	15
	Water	150	Isooctanol	15
F	Methanol	165	Chloroform	15
	Water	135	Heptane	5

Hostalene prepared by the method of Bergström *et al.* (*7*) was used as the supporting material for the stationary phase. Chromatograms were run at a constant temperature of 23°C. The effluent was titrated with 0.02 N methanolic NaOH, and suitable aliquots were assayed for the radioactivity. The radioactivity was counted as infinitely thin layer in a proportional gas flow counter made in Shimazu Company, Japan. The total recovery of radioactivity in the bile was 6.6 per cent.

Chromatography of the Bile Acid Fraction—Isolated bile acid was chromatographed with phase system F (Fig. 1). In Fig. 1, the first and second peaks show the same elution volume as cholic acid (*10*) and trihydroxycoprostanic acid (*11*) respectively. Fractions containing the peak II were combined and the solvent was evaporated off. The residue was dissolved in water and acidified with dilute HCl and extracted with ether. The ether extract was washed with water and evaporated to dryness. The residue was mixed with 5 mg. of unlabeled trihydroxycoprostanic acid and rechromatographed with phase system F (Fig. 2). The residue obtained from the fraction containing peak I was treated similarly, mixed with 5 mg. of cholic acid and chromatographed with phase system C (Fig. 3).

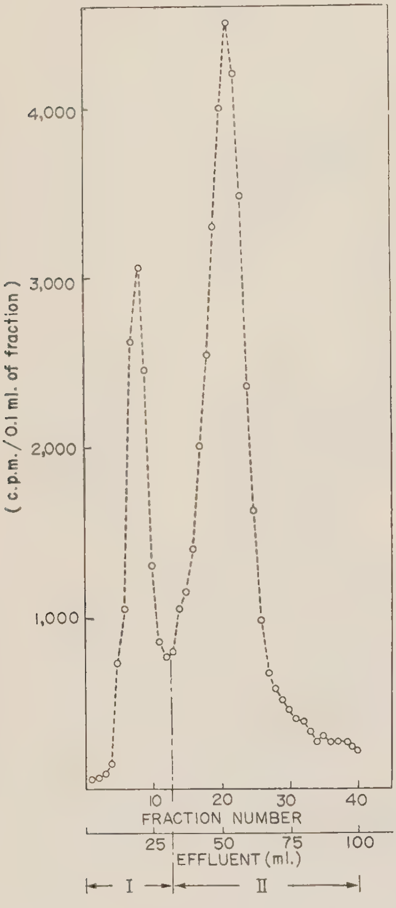

Fig. 1. Column chromatography of the hydrolyzed bile acid isolated from bull frog bile after injection of cholesterol-4-C^{14}. Column, 9 g. of Hostalene; phase system F.

As shown in Figs. 2 and 3, titration curve of trihydroxycoprostanic acid and cholic acid were clearly coincided with the curve of radioactivity. The identity of the radioactive compounds with trihydroxycoprostanic acid and cholic acid respectively was further strengthenedby isotope dilution as shown in the following tables. The effluents of Fig. 2 were combined and the solvents were evaporated off. The residues were dissolved in water, acidified with dilute HCl and extracted with ether. The ether extract was washed with water and evaporated to dryness. One hundred milli- grams of authentic trihydroxycoprostanic acid were added to this residue and then recrystallized four times from different solvents (Table I). By the same method, 30 mg. of cholic acid were added to theacid obtained from the band of Fig. 3 and recrystallized (Table II).

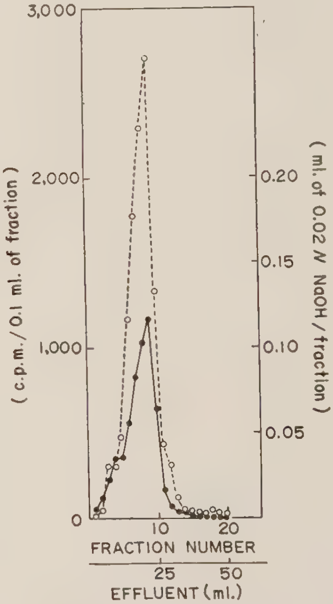

Fig. 2. Rechromatography of the second radioactive band shown in Fig. 1, II mixed with unlabeled trihydroxycoprostanic acid. Column, 4.5 g. of Hostalene, phase system F; solid line, titration values; broken line, radioactivity.

The identify of the labeled compounds with trihydroxycoprostanic acid and cholic acid respectively was further confirmed by the method of paper chromatography of Sjövall (*12*), which was carried out using systems as followings: mobile phase, isopropyl ether/heptane 60:40, stationary phase, 70% acetic acid/water (Fig. 5) and mobile phase, ethylene chloride/heptane 60:40; stationary phase, 70% acetic acid/water (Fig. 4), and the spots were detected by the reaction with phosphomolybdic acid. R_f values of bile acids separated from the band I and II of Fig. 1 were identical to that of authentic trihydroxycoprostanic acid (*1*) and cholic acid (*13*) respectively as shown in Fig. 4 and 5. Radioactivity was detected by radioautography on the spots corresponding to that of authentic acids.

A portion of the radioactivity in the ori-

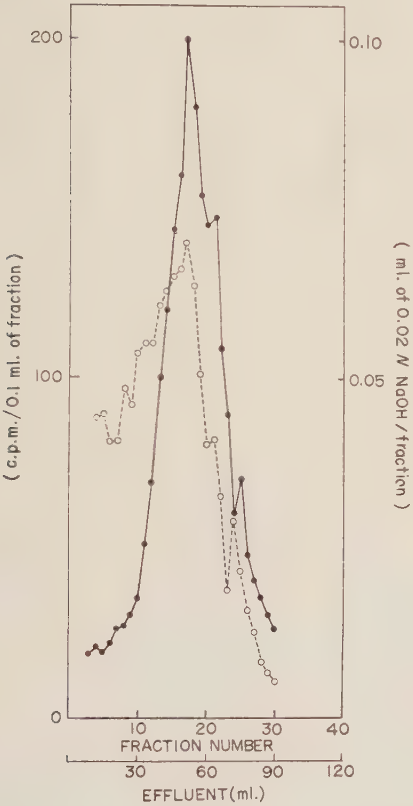

FIG. 3. Rechromatography of the first radioactive band of Fig. 1, I mixed with unlabeled cholic acid. Column, 4.5 g. of Hostalene; phase system C; solid line, titration values; broken line, radioactivity.

TABLE I

Successive Crystallization of Trihydroxycoprostanic acid-4-C^{14} from Various Solvents

Solvent	Specific activity (c.p.m./mg.)
Ethyl acetate	1200
Acetone	941
Ethyl acetate	900
Acetone	900

TABLE II

Successive Crystallization of Cholic Acid-4-C^{14} from Various Solvents

Solvent	Specific activity (c.p.m./mg.)
Methanol-water	60
Ethanol-water	63
Acetone-water	67
Methanol-water	65

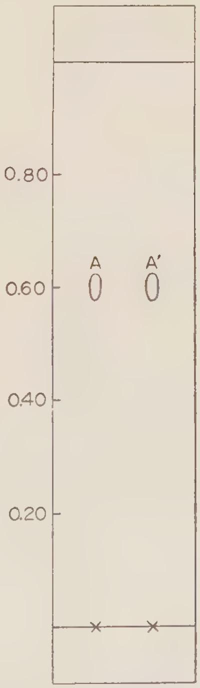

FIG. 4. Paper chromatography of bile acid obtained from the fraction corresponding to curve I of Fig. 1. A: Cholic acid; A′: Bile acid obtained from the fraction corresponding to curve I.

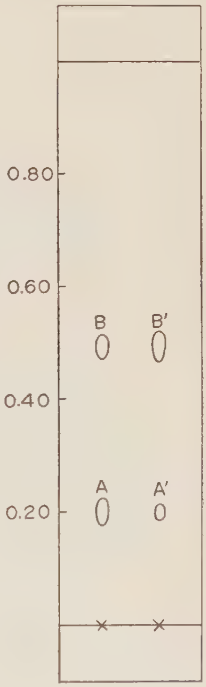

FIG. 5. Paper chromatography of bile acid obtained from the fractions corresponding to curve I and II of Fig. 1. A: Cholic acid, B: Trihydroxycoprostanic acid. A′, B′: Bile acid of curve I and II.

ginal bile acid extract was retained in the stationary phase of the column. The column was extracted with 95% alcohol and alcohol extract was evaporated to dryness. The residues obtained were chromatographed with phase A (Fig. 6). The main band was similar to that of lithocholic acid. Therefore, a main portion of radioactive fraction was mixed with 5 mg. of lithocholic acid and rechromatographed with phase system A (Fig. 7). The curves of Fig. 6 show that the radioactive compound is different from lithocholic acid. The specific radioactivity of cholic acid, trihydroxycoprostanic acid and the unidentified acid are shown in the Table III.

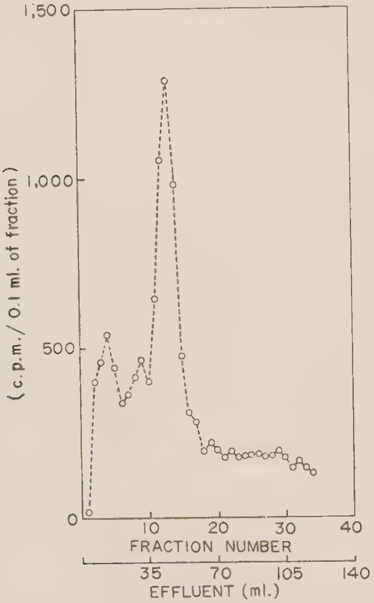

Fig. 6. Column chromatography of labeled material retained in the stationary phase of the column shown in Fig. 1. Column, 4.5 g. of Hostalene; phase system A; broken line, radioactivity.

Table III

Specific Radioactivity of the Bile Acids Isolated from Bile of Bull Frog

Bile acid	Specific activity (c.p.m./mg.)
Cholic acid	44,470
Trihydroxycoprostanic acid	107,880
Unknown acid	55,886

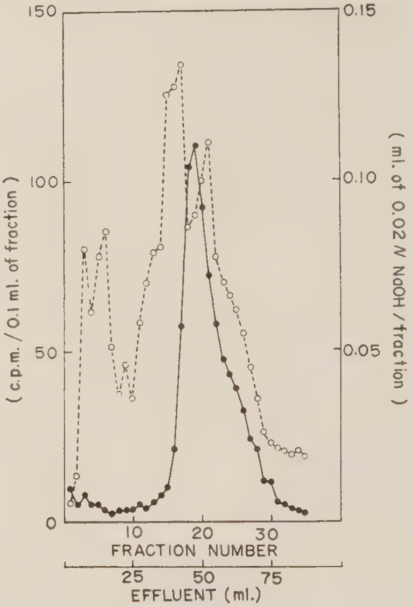

Fig. 7. Rechromatography of a mixture of unlabeled lithocholic acid and that obtained from the radioactive band shown in Fig. 6. Column, 4.5 g. of Hostalene; phase system A; broken line, radioactivity; solid line, titration values.

Column Chromatography of the Bile Sterols Fraction—The isolated bile sterol fraction was chromatographed with phase system A (Fig. 8). The radioactivity of effluent volumes of 100 ml. gave six peaks and specific radioactivity of each band is shown in the Table IV. Each peak of the radioactivity corresponded to the curve of chromatography of normal

Table IV

Specific Radioactivity of Bile Sterols Isolated from Bile of Bull Frog

Bile sterol	Specific activity (c.p.m./mg.)
Band I	7,130
Band II	18,400
Band III	17,718
Band IV	16,600
Band V	28,868
Band VI	29,942

bile sterol in bull frog*. Further work on this bile sterol is in progress.

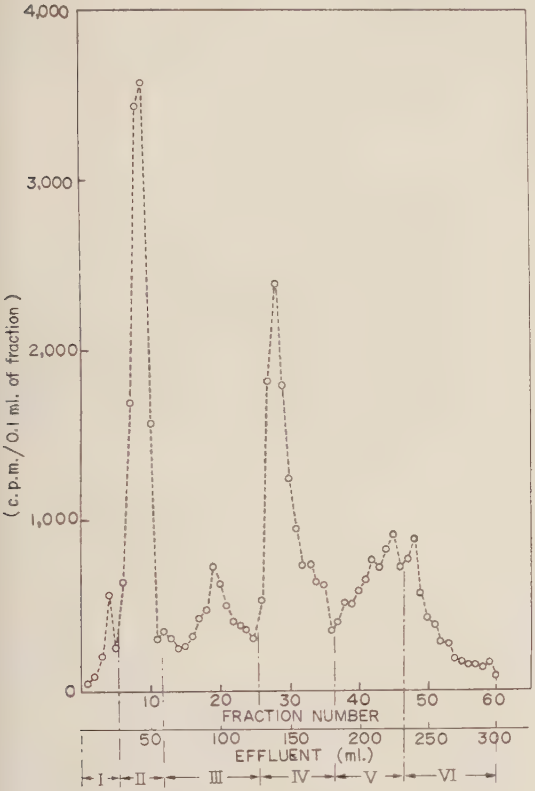

FIG. 8. Chromatographic separation of hydrolyzed bile sterols isolated from bile of bull frog after injection of cholesterol-4-C^{14}. Column, 13.5 g of Hostalene; phase system A;

Paper Chromatography of the Bile Acids Isolated from Normal Bull Frog—Bile obtained from six normal gall bladders was hydrolyzed with 2.5 *N* NaOH by the usual method. The hydrolysate was poured into a large amount of water to separate the bile sterol and then filtered. After acidification of the filtrate, the bile acid was extracted with ether. The ether extract was washed with water to neutral and concentrated to a small volume. The results of paper chromatography of this bile acid mixture using two kinds of phases are shown in Fig. 9.

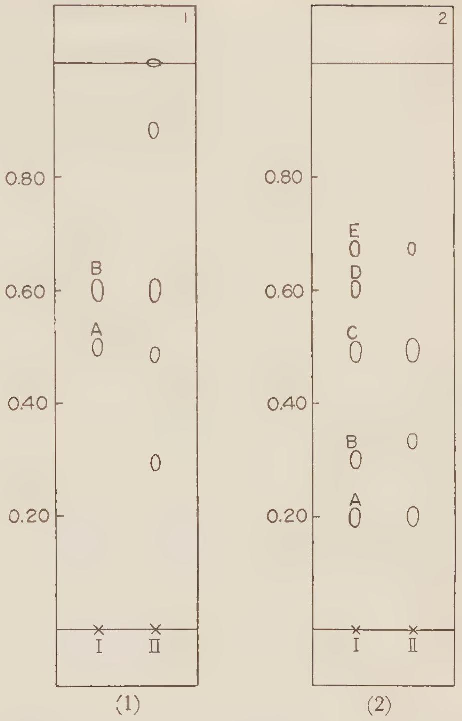

FIG. 9. Paper chromatography of bile acids obtained from normal bile.

(1) Mobile phase, ethylene chloride/heptane 60:40; stationary phase, 70% acetic acid/water. I: Authentic compound. A: Nor-cholic acid. B: cholic acid. II: Bile acid of bull frog.

(2) Mobile phase, isopropyl ether/heptane 60:40; stationary phase, 70% acetic acid/water. I: Authentic compounds. A: cholic acid. B: homocholic acid. C: trihydroxycoprostanic acid. D: chenodeoxycholic acid. E: deoxycholic acid. II: Bile acid of bull frog.

DISCUSSION

Three kinds of bile acids and several bile sterols were isolated from the bile of bull frog previously injected with cholestero-l4-C^{14}. The first and the second radioactive compouunds as shown in curve of Fig. 1 were identified with cholic acid and trihydroxycoprostanic acid respectively.

Formation of cholic acid from cholesterol has been encountered in several mammalian species, rat (*14*), mouse (*15*) and rabbit (*16*).

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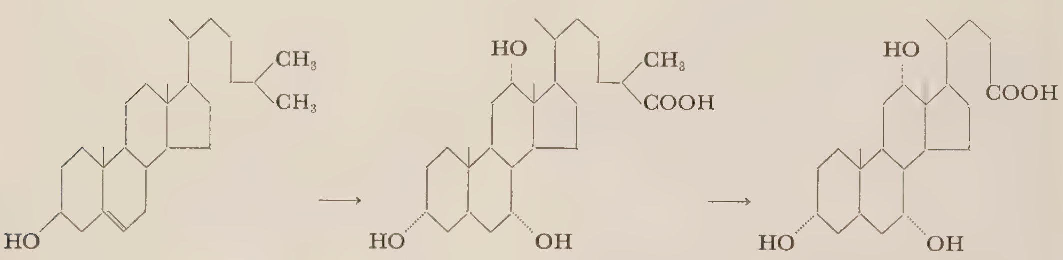
* Kazuno, T., Masui, T., and Hoshita, T., *J. Biochem.*, in press.

Formation of cholic acid from trihydroxycoprostane or trihydroxycoprostanic acid has been verified in the laboratory of S. Bergström (*17*, *18*), and of T. Kazuno (*5*, *19*). Recently M. W. Whitehouse (*11*) observed the conversion of cholesterol-27-C^{14} to trihydroxycoprostanic acid-27-C^{14} in alligater. From the author's experiment it is evident that trihydroxycoprostanic acid is an intermediate substance in the conversion of cholesterol to cholic acid as shown in Diagram I. The unidentified acid separated from lithocolic acid fraction is likely to be dihydroxycoprostanic acid, because this is less hydrophobic than that of trihydroxycoprostanic acid.

In this experiment, moreover, it is verified that a small amount of cholic acid and desoxycholic acid were contained in normal bull frog bile.

DIAGRAM I



SUMMARY

After injection of cholesterol-4-C^{14} to normal bull frog, radioactive bile sterols were found in bile as the main metabolites and beside them, radioactive trihydroxycoprostanic acid and cholic acid were identified.

The author wishes to express her deep gratitude to Prof. T. Kazuo for his kind advice throughout this work, and express her thanks to Prof. S. Bergström of the Karolinska Institute who has kindly given Hostalene.

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States of Tyrosine Residues in the Molecules of Insulin, Lysozyme and Catalase

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The absorption band of tyrosine is known to shift from 275 to 293 mμ with intensification, when the phenolic group is caused to ionize into phenoxide ion by the effect of alkali. Spectrophotometric titration based upon this phenomenon can be applied to the study of the states of tyrosine residues in protein molecules, since under similar conditions other amino acids show, if any, only small changes in absorption hands in the wavelength range of the tyrosine bands.

The method of spectrophotometric titration based on this principle was first applied by Crammer and Neuberger (*1*) to the investigation of the state of tyrosine residues in egg albumin, in which they found strongly bound tyrosine residues which did not ionize unless the molecule was denatured by exposure to extremely high pH, 13.0. They also observed an anomalously high pK, 11.0, for the tyrosine residues in insulin as compared with 10.0～10.1 (*2–4*) for that of tyrosine. Extensive studies along similar lines have since been made by various workers on serum albumin, ribonuclease and lysozyme. Tanford and Roberts, Jr. (*5*) observed the transformation of tyrosine band of bovine serum albumin and concluded that the phenolic groups may be hydrogen-bonded in the native molecule. Ribonuclease was studied by Shugar (*6*), who reported that 60 per cent of the tyrosine residues ionize instantaneously with alkali, while the rest of them ionize only after denaturation. Later studies by Tanford and his co-workers (*7*) showed that in the ribonuclease molecule three out of six tyrosine residues are embeded in non-polar portions of the molecule and ionize slowly and irreversibly, and that the remaining three ionize instantaneously and reversibly at the surface of the molecule. Fromageot and Schnek (*8*) observed a reversible transformation of the tyrosine band in lysozyme with a normal ionization curve of the first-order sigmoid shape, while Tanford and Wagner (*9*) obtained an ionization curve which was flatter than the first-order sigmoid curve or than would be expected from the simple ionization mechanism of one kind of tyrosine residues.

These data obtained by spectrophotometric titration have revealed the existence of at least two types of tyrosine residues in proteins, one being instantaneously ionizing residues which are similar in ionization property to the free tyrosine molecule, and the other being bound residues which are hydrogen-bonded or embeded in the interior of protein molecules and capable of ionizing only slowly or after denaturation.

The present paper deals with a similar line of research made on the states of tyrosine residues in insulin, lysozyme and catalase. By closer analysis of the results obtained we were able to clarify not only the number of different types of tyrosine residues but also some detailed aspects of their ionization characteristics. Throughout this report, the two types of tyrosine residues mentioned above will simply be called 'free' and 'bound' residues.

EXPERIMENTALS

Materials—Crystalline beef insulin was kindly supplied by Dr. T. Homma of Shimizu Pharmaceutical Company. It was a recrystallized sample prepared by the method of Romans, Scott and Fisher (*10*) as modified by Sawada, Shibata and Itani (*11*). Lysozyme crystals were prepared from hen's egg white by the Alderton and Fevold method (*12*). Crystalline catalase was obtained from bovine liver by the Shirakawa method (*13*), using ammonium sulfate for separation and recrystallizing several times by changing the pH of the solution. The concentration of catalase was determined spectroscopically from the height of the Soret band at 405 mμ, assuming its molar extinction coefficient (ε) to be 3.4×10^5 (*14*). The concentration of insulin and lysozyme were determined gravimetrically, assuming their molecular weights to be, respectively, 5734 (*15*) and 15,000, in which the latter is a mean value obtained from various data (*16–19*).

Spectroscopic Observation—Observations of the tyrosine bands and their changes by treatment with alkali were carried out using a Cary Recording Spectrophotometer Model 14 *M*. To observe the spectral changes, protein solutions of a given alkaline pH and of pH 7.0 were separately placed in the sample and reference compartments, and the difference in absorbance (designated as ΔE) between these solutions was directly recorded against time at 295 or 298 mμ; 295 mμ was the maximum position of the positive peak in the difference spectra of tyrosine, insulin and lysozyme, and 298 mμ was the corresponding maximum position for catalase. The recording was started 15 second after making a neutral protein solution alkaline with 1.0 *M* KOH and 1.0 *M* KCl, the salt being used to keep the ionic strength of the sample solution constant at $\mu=0.56$. After the transformation of band was equilibriated or completed, the difference spectrum between the alkaline and neutral solutions was observed. Similar measuremens were carried out with proteins in 4 *M* guanidine solution in order to see the effect of denaturation by the reagent (*20*). All measurements were carried out at room temperature, 15–17°C.

Analysis of Results—ΔE values given in this paper (at 295 mμ for insulin and lysozyme, at 298 mμ for catalase) are those calculated in terms of the number of tyrosine residues per molecule of protein, assuming the $\Delta\varepsilon$ value (change of the molar extinction coefficient due to ionization) to be 2305, which is the value determined in the present study for amino acid tyrosine at 295 mμ. From the ionization curve (ΔE-pH curves) of different tyrosine residues observed separately by the procedures described later, determinations were made of the pK value and the number (m) of hydroxyl ion, defined by:

$$\mathrm{TyrH}+m\mathrm{OH} \rightleftharpoons \mathrm{Tyr} \qquad (1)$$

where TyrH and Tyr stand, respectively, for non-ionized and ionized forms of tyrosine residue. The number m is equal to the order of sigmoid curve of ionization, which can be more than one if the hydroxyl ions undergo some additional reactions before the final step of ionization. Such additional reactions are thought to take place when hydrogen bonds are split to give rise to 'free' tyrosine residues. On the other hand, if the splitting of hydroxyl ion represents the rate-determining step of the overall reaction, the number of hydroxyl ions involved would be equal to or smaller than m determined under equilibrium condition. The number of hydroxyl ions involved in such cases, which may be designated by n in distinction to m, may be determined from the pH dependency of the rate of ionization, assuming the following reaction:

$$\mathrm{TyrH}+n\mathrm{OH} \xrightarrow{k} \mathrm{Tyr} \qquad (2)$$

$$-\frac{d\,(\mathrm{TyrH})}{dt} = k\,(\mathrm{OH})^n(\mathrm{TyrH}) \qquad (3)$$

where k is the rate constant. If the fraction (f) of non-ionized forms is defined by

$$f=\frac{(\mathrm{TyrH})}{(\mathrm{TyrH})+(\mathrm{Tyr})} \qquad (4)$$

we obtain from equation (3) the following relation:

$$\log f = -0.434 \times k\,(\mathrm{OH})^n t \qquad (5)$$

Therefore, by plotting $\log f$ against reaction time we can determine the value of $0.434 \times k\,(\mathrm{OH})^n$, and the pH dependency of the value enables us to determine the value of k as well as the number n.

RESULTS

Tyrosine—In a preliminary experiment, the spectral and ionization characteristics of tyrosine were studied. Non-ionized tyrosine solution of pH 7.0 and its completely ionized solution of pH 13.0 showed an absorption band at 275 and 293 mμ with ε values of 1275 and 2325, respectively. On adding alkali to the neutral solution, complete transformation of the band occurred instantaneously. The difference spectrum (curve A in Fig. 1) between the neutral and alkaline solutions showed a negative peak at 273 mμ and a positive peak at 295 mμ with a $\Delta\varepsilon$ value of 2305.

The ΔE value at 295 mμ was observed, varying the pH of the alkaline sample between 9.0 and 13.0. The ionization curve thus obtained (curve A in Fig. 2) showed a pK value of 10.0 with $m=1.0$ which is in agreement with the pK value, 10.0–10.1, reported by various workers (*2–4*). The ionization curve of tyrosine in 4 M guanidine solution (curve B in Fig. 2) was practically the same, both in shape and in pK value, as that obtained without guanidine. This similarity indicates that the differences described later for the tyrosine bands of proteins with and without guanidine treatment are attributable to the denaturation of proteins caused by guanidine.

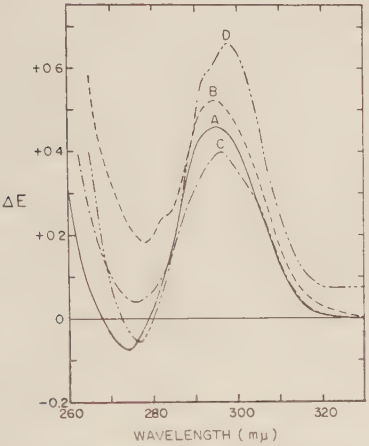

Fig. 1. Difference spectra between alkaline and neutral solutions of tyrosine and proteins.
A: tyrosine, in concentration of $2.00\times10^{-4}M$.
B: insulin, in concentration of $0.546\times10^{-4}M$.
C: lysozyme, in concentration of $0.540\times10^{-4}M$.
D: catalase, in concentration of $0.297\times10^{-5}M$.

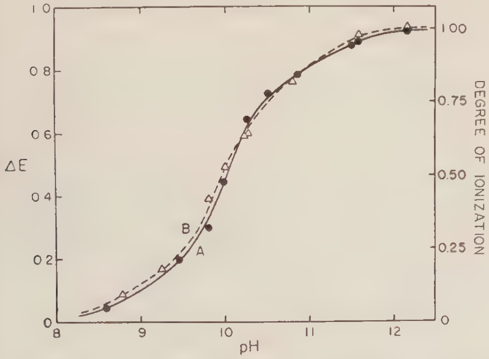

Fig. 2. Ionization curves of tyrosine. Concentration: $4.00\times10^{-4}M$.
A: without guanidine. B: with guanidine.

Insulin—On adding alkali to a neutral solution of insulin, the transformation of the band proceeded in two steps; *i.e.* an instantaneous but partial transformation and a follow-up gradual transformation which required roughly 2 hours to be completed. Curve B in Fig. 1 is the difference spectrum between insulin solutions of pH 13.0 and 7.0, of which the alkaline sample had been left standing for 3 hours before observation to ensure the completion of ionization. From the ΔE value obtained at 295 mμ, the number of tyrosine residues per molecule of insulin was calculated to be 4.13 which is close to the number 4.0 determined by chemical analysis (*21*). The 3 per cent deviation from the round number is probably due to a slight overlapping of the band appearing in curve B at shorter wavelengths. When the comparison of the two solutions was made immediately after the addition of alkali, the ΔE value was found to be approximately three fourths the value observed after complete ionization. Repeated measurements at 295 mμ gave us 3.05 as the number of the instantaneously ionizable or 'free' tyrosine residues per molecule of insulin, so that the remaining one is inferred to be of the 'bound' type.

The ΔE value at 295 mμ was measured as a function of pH, both immediately and 3 hours after the preparation of the alkaline samples. The ionization curve thus obtained are shown by curves A and B in Fig. 3. Curve A shows the pK value of 10.4 with $m=1.0$. This pK value is slightly larger than that for tyrosine, indicating that even the 'free' residues may be more or less weakly bound. The value of m for curve B is appreciably smaller than 1.0, suggesting that the curve is a composite of ionization curves with different pK's. The subtraction of curve A from curve B gave curve C which may be regarded as the ionization curve of the bound residue. The m value for curve C was 1.0 and its pK value was 11.4 which is far greater than the pK value for tyrosine, indicating the strongly bound states of the residue in question. The process of ionization of the 'bound' residue was followed, by measuring the change with time of the ΔE value at 295 mμ. The result is shown by line A in Fig. 4 where f is the fraction of non-ionized forms of the 'bound' residues in solution. The linear relationship between log f and time t indicates that the rate is proportional to the concentration of TyrH, obeying equation (3). From the inclination of line A in Fig. 4 and the similar data at other pH's, the value of $0.434 \times k(\mathrm{OH})^n$ was calculated by equation (5) as a function of pH. Plot of log $k(\mathrm{OH})^n$ against pH gave a linear relationship with $n=1$, which is shown by line A in Fig. 5. The value of k calculated from the intersection of the line at the ordinate was 3.4×10^{-3} M^{-1} sec.$^{-1}$

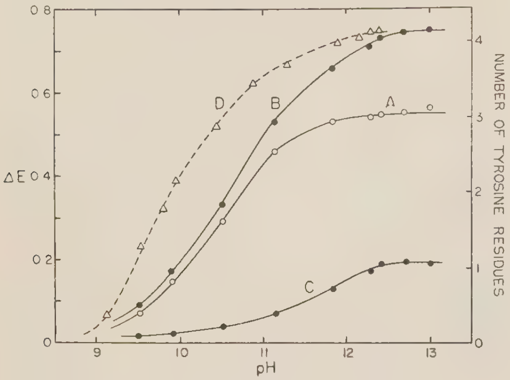

FIG. 3. Ionization curves of tyrosine residues in insulin. Concentration: $0.780 \times 10^{-4} M$.

A: plotting of ΔE which was determined immediately after the shift of pH caused by the addition of alkali. This curve represents the ionization curve of 'free' tyrosine residues.
B: plotting of ΔE which was determined after equilibration of the change at each pH.
C: theoretical ionization curve of 'bound' tyrosine residue drawn by assuming pK=11.4 and m=1.0. Solid circles: the values obtained by subtracting the data obtained on curve A from those on curve B.
D: ionization curve of insulin in 4 M guanidine solution.

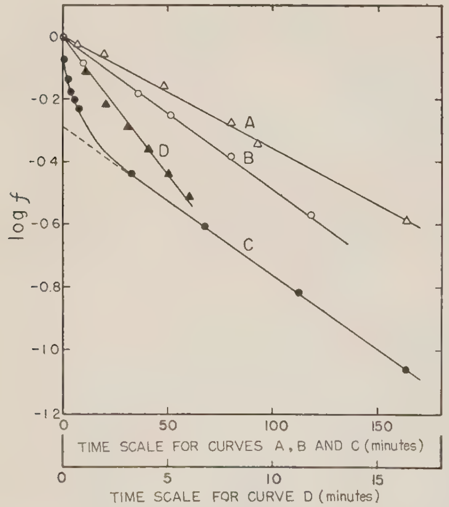

FIG. 4. Plotting against reaction time of the values of log f obtained for 'bound' residues of insulin, lysozyme and catalase.

A: insulin, pH 12.42.
B: lysozyme, pH 13.75.
C: catalase, 'strongly bound' residues, pH 11.02.
D: catalase, 'weakly bound' residues, pH 11.02.

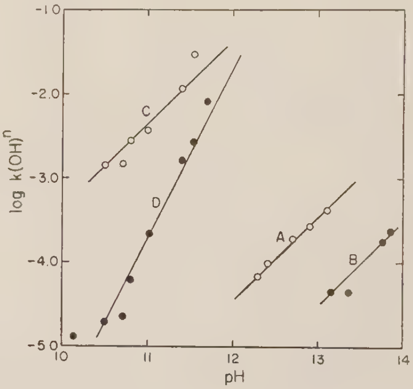

FIG. 5. Plotting against pH of log $k(\mathrm{OH})^n$ obtained for insulin, lysozyme and catalase.

A: insulin, pH 12.42.
B: lysozyme, pH 13.75.
C: catalase, 'weakly bound' residues.
D: catalase, 'strongly bound' residues.

When insulin was dissolved in 4 M guanidine solution, an entirely different phenomenon was observed; namely, for all 4 tyrosine residues, the transformation of band caused by alkali occurred instantaneously and no further change was observed later. The ionization curve obtained in the presence of guanidine is shown by curve D in Fig. 3, which is practically the same as that obtained for tyrosine (Fig. 2). The marked decrease of the pK value effected by the addition of guanidine indicates that the reagent might have split the hydrogen bond directly related to the 'bound' residue or might have unfolded the insulin molecule to make the residue exposed.

Lysozyme—Instantaneous and gradual transformations of the tyrosine band were also observed with lysozyme. In this case, however, the second transformation was slower than that observed with insulin, and it took roughly 4 hours to obtain a constant reading of ΔE. Curve C in Fig. 1 is the difference spectrum between lysozyme solution of pH 13.7 and 7.0, which was observed 5 hours after the preparation of the alkaline sample. The ionization curves obtained immediately and 5 hours after the preparation are shown by curves A and B, respectively, in Fig. 6. As may be seen, the ΔE values obtained in these two cases were the same below pH 12.0 but different above the pH value. Curve A reached the ΔE level corresponding to 2 tyrosine residues per molecule of lysozyme, whereas curve B obtained 5 hours later approached the level of 3 residues per molecule, which is equal to the number determined by chemical analysis (*22*). It is, therefore, evident that 2 out of 3 tyrosine residues in the molecule are 'free' and the remaining one is 'bound'. The pK value of the 'free' residues estimated from curve A was 10.5, which is close to the value 10.4 obtained for the 'free' residues of insulin.

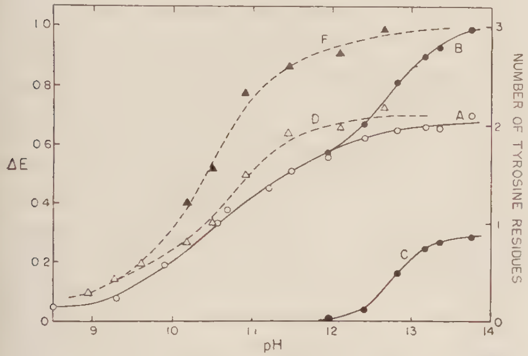

FIG. 6. Ionization curves of tyrosine residues in lysozyme. Concentration: $1.491 \times 10^{-4} M$.

A: plotting of ΔE which was determined immediately after the shift of pH caused by the addition of alkali. This curve represents the ionization curve of 'free' tyrosine residues.

B: plotting of ΔE which was determined after equilibration of the change at each pH.

C: theoretical ionization curve of 'bound' tyrosine residue drawn by assuming pK=12.8 and $m=2.0$. Solid circles: the values obtained by subtracting the data obtained on curve A from those on curve B.

D: plotting of ΔE in the presence of 4 *M* guanidine, which was determined immediately after the shift of pH caused by the addition of alkali.

F: plotting of ΔE in the presence of 4 *M* guanidine, which was determined after equilibration of the change at each pH.

The ionization curve of the 'bound' residue is shown by curve C which was obtained by subtraction of curve A from curve B. The pK value determined from curve C was 12.8, a value which is even higher than the pK value obtained for the 'bound' residue of insulin. Another conspicuous difference from the results obtained with insulin lies in the value of m which was found to be almost exactly equal to 2.0 for lysozyme.

The ionization rate constant for the 'bound' residue was determined in the same way as before. The plot of log f against time gave a straight line as shown by line B in Fig. 4, from which the value of $0.434 \times k\,(OH)^n$ was evaluated. Line B in Fig. 5 shows a linear relationship between log $k(OH)^n$ and pH with $n=1.0$. It is, therefore, clear that one hydroxyl ion was involved in the rate-determining step of ionization, while two hydroxyl ions ($m=2.0$) were required for the complete ionization. From the intersection of line B at the ordinate of Fig. 5, the value of k was estimated, with the aid of equation (5), to be $3.2 \times 10^{-4}\ M^{-1}\ \text{sec.}^{-1}$

The strongly bound state of the one tyrosine residue in lysozyme is reflected also in the result obtained with 4 *M* guanidine. Curves D and F in Fig. 6 are the ionization curves obtained at two observation times after the addition of guanidine. Whereas the 'bound' residue in insulin was transformed completely into the 'free' type by the action of 4 *M* guanidine, the transformation is incomplete in the case of lysozyme, although the pK value was larglely shifted toward lower pH.

Catalase—The molecular weight of catalase is 225,000~248,000 (*23–26*), being much higher than those of insulin and lysozyme molecule. The content of tyrosine is 6.80 per cent in weight according to the chemical analysis (*27*), which corresponds to 93 tyrosine residues per molecule when the molecular weight is assumed to be 248,000 (*24*, *26*). The ionization of the tyrosine residues in catalase molecule is of special interest, since the native molecule has been found to split partially between pH 9.9 and 11.0 and completely at pH 12.0 into three subunits (*26*, *28*) which are indistinguishable from each other by sedimentation analysis. Difference spectrum between alkaline and neutral solutions of catalase shows a positive peak at 298 mμ, which is located slightly redward as compared with the corresponding peaks of the other proteins studied (curve D in Fig. 1). The number of tyrosine residues was calculated from the ΔE value at 298 mμ, assuming the same $\Delta\varepsilon$ value as determined for tyrosine at 295 mμ. The number thus determined was 93 per molecule which is in excellent agreement with the number calculated above based on the chemical analysis. This agreement shows that the error due to the assumptions made for the $\Delta\varepsilon$ value was very small.

The process of ionization was investigated by following the change with time of ΔE at 298 mμ. Also in this case, the change of the tyrosine band occurred in two steps, one instantaneously and the other slowly. However, the second process proceeded much more rapidly, especially at the beginning, as compared with the similar processes observed with insulin and lysozyme. The initial rate in the second step increased so greatly with the increase of pH that we could follow only a part or none of the transformation at pH's higher than 11.6. Curves A and B in Fig. 7 show, respectively, the ionization curve observed 15 second after the preparation of the alkaline samples and that obtained after the transformation had been equilibriated. Curve A has an irregular shape and joins with curve B above pH 12.1. The portion of curve A below pH 11.6 appears to follow a first-order sigmoid curve. This fact as well as the hight initial rate of the second process suggests that the process had proceeded partially between pH 11.6 and 12.1 and completely above pH 12.1, within 15 second after the preparation of alkaline samples. Curve C, which was obtained by subtraction of curve A from curve B, represents the ionization curve of the 'bound' residues. The height of the flat level of curve C between pH 11.0 and 11.6 indicates that approximately 26 residues are of the 'bound' type. The remaining 67 residues may, therefore, be inferred to be of the 'free' type. The number m determined from curve C was 1.4, an indication that there exist more than one type of residues with different ionization properties. The apparent pK value determined from curve C was 10.5. The dashed curve shown

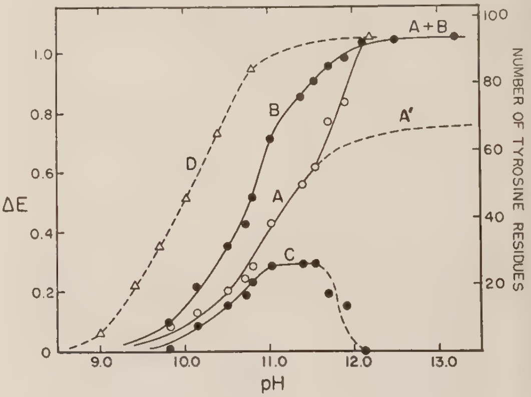

FIG. 7. Ionization curves of tyrosine residues of catalase. Concentration: $0.478\times10^{-5}M$.

A: plotting of ΔE which was determined immediately after the shift of pH caused by the addition of alkali.

B: plotting of ΔE which was determined after equilibration of the change at each pH.

C: ionization curve of 'bound' tyrosine residues which was obtained by subtracting curve A from curve B.

D: ionization curve in 4 M guanidine solution.

A': theoretical ionization curve of 'free' tyrosine residues drawn by assuming pK=11.0 and $m=1.0$.

by A' in Fig. 7 and the part of solid curve A below pH 11.6 were drawn according to the calculation for 67 residues with assumed pK value of 11.0. As may be seen from the figure, the theoretical values of ΔE thus calculated agreed nicely with the experimental values below pH 11.6.

The 'bound' tyrosine residues in catalase may be further divided into two different types. Curve C in Fig. 4 shows an example of the decay process of the fraction *f* of non-ionized forms observed at pH 11.0. The value of log *f* decreased rapidly in earlier stages but the rate dropped down later when a linear relationship was obtained between log *f* and the reaction time *t*. This result revealed the presence of two types which we shall call 'weakly' and 'strongly bound' residues. The linear relationship obtained in the later stage may be accounted for by the ionization of the 'strongly bond' residues, which occurred after the complete ionization of the 'weakly bound' residues in the earlier stage.

The extrapolation (dashed line in Fig. 4) of the linear part in the later stage will show the time course of ionization only of the 'strongly bound' residues, and its intersection at the ordinate will give us the fraction of the 'strongly bound' residues in the total bound residues, the amount of which can be evaluated from the data at completely ionizing pH between 11.4 and 11.6. The number of the 'strongly bound' residues thus determined was 19 ± 1, indicating that 7 ± 1 residues were of the 'weakly bound' type. From the hypothetical values of log *f* on the extrapolated dashed line in Fig. 4, the fraction *f* of the 'strongly bound' residues was calculated as a function of reaction time. By subtracting the fractions thus obtained from the fraction of the total bound residues, the values of log *f* for the 'weakly bound' residues were calculated. The process of ionization of the 'weakly bound' residues is shown by line D in Fig. 4, which proved that equation (3) holds also for these residues. From the inclination of the line as well as from similar data at other pH's, the value of $0.434 \times k(\mathrm{OH})^n$ at various pH's was calculated. The linear relationship between $\log k(\mathrm{OH})^n$ and pH (curve C in Fig. 7) showed that the 'weakly bound' residues ionize with the rate constant $k = 4.5\,M^{-1}\mathrm{sec}^{-1}$ and $n = 1.0$. The pH dependency of $\log k(\mathrm{OH})^n$ for the 'strongly bound' residues which was calculated from the linear drop of log *f* in the later stage is shown by curve D in Fig. 7, from which we obtain $n = 2.0$ and $k = 2.0 \times 10^2\ M^{-2}\mathrm{sec}^{-1}$.

Curve D in Fig. 7 shows the ionization curve obtained in the presence of 4 *M* guanidine. In this case, all of the residues ionized instantaneously and completely on the addition of alkali, showing the values of pK = 10.0 and $m = 1.0$. It may be concluded that the residues in the catalase molecure which we have called "strongly bound residues" are subjected to a force much weaker than that associated with the 'bound' residue in lysozyme.

DISCUSSION

The number of various types of tyrosine residues determined in this study are listed in Table I together with their ionization characteristics. The number of the 'bound residue' in insulin agrees with the result reported by Scheraga (*29*, *30*) who located its position in the B chain of the insulin molecule based on the difference spectrum between insulin solution before and after trypsin digestion. This agreement in number obtained by the two different techniques as well as in the pK value, 11.4, for the 'bound residue' seems to indicate that the pK value, 11.0, reported by Crammer and Neuberger (*1*) may have been an apparent value averaged for the 'free' and 'bound' residues. The question raised by Tanford and Wagner (*9*) as to the flat ionization curve of lysozyme may be answered by the influence from the present study that only one residue out of the total three is in 'bound' form. The 'strongly bound' nature of this residue may, possibly, have been the reason why Fromageot and Schnek (*8*) were not able to detect this residue.

The high pK value, 11.4, for the bound

TABLE I

Ionization Characteristics of Tyrosine Residues in Insulin, Lysozyme and Catalase

(k value is expressed in $M^{-2}\text{sec}^{-1}$ for the 'strongly bound' residues of catalase and in $M^{-1}\text{sec}^{-1}$ for the other residues.

	number	pK	m	k	n
Insulin	4				
'free'	3	10.4	1	instantaneous	—
'bound'	1	11.4	1	3.4×10^{-3}	1
Lysozyme	3				
'free'	2	10.5	1	instantaneous	—
'bound'	1	12.8	2	3.2×10^{-4}	1
Catalase	93				
'free'	67	11.0	1	instantaneous	—
'weakly bound'	7	—	—	4.5	1
'strongly bound'	19	—	—	2.0×10^{2}	2

residue of insulin indicates the presence of a hydrogen bond involved in its ionization. The value of both m and n was 1.0, which suggests that there was no additional reaction involved before the ionization process. It may, therefore, be inferred that the hydrogen atom attached to the phenolic group of the 'bound' residue in insulin is hydrogen-bonded, and that its splitting results directly in the ionization of the residue. In the case of the lysozyme molecule, the value of m was 2.0 whereas n was 1.0. This difference may be accounted for by assming two consecutive reactions, each requiring one hydroxyl ion, in which the earlier reaction proceeds more slowly than does the later reaction. Probably, the earlier reaction is the process of splitting of a hydrogen bond and the later reaction is the ionization of the 'free' residue thus formed.

The tyrosine residues in catalase were found to be of three types; one 'free' and two differently 'bound' types. The apparent pK value, 10.5, for the two types of 'bound' residues is in the pH range, in which the native catalase molecule has been found to split into subunits (*26*, *28*). The splitting will naturally expose the residues located in the interior of the native molecule or between the subunits. The rather rapid ionization (with $n=1.0$) of the 'weakly bound' residues seems to be an indication that the residues may be those located in the interior without being bound by hydrogen bonds. On the other hand, the value of $n=2.0$ found for the 'strongly bound' residues indicates that the gradual ionization was due to the presence of hydrogen bonds and not to the dissociation. In other words, the 'strongly bound' residues may be hydrogen-bonded in the interior or at the surface of the catalase molecule.

SUMMARY

The states of tyrosine residues in insulin, lysozyme and catalase were studied by spectrophotometric titration based upon the shift with intensification of their tyrosine bands due to the ionization of the phenolic group. On adding alkali to the protein solutions, the transformation of the band occurred in two steps, and instantaneous but partial transformation and a follow-up gradual transformation, from which we could distinguish two types, named 'free' and 'bound', of tyrosine residues according to their susceptibility to ionization caused by alkali. The number of these different types of residues in those proteins as well as their ionization characteristics (pK value, rate constant and the number of hydroxyl ion involved in the ionization) was determined by analysing the ionization curves and by measuring the rates of ionization and their pH dependency. It was revealed from these measurements that, in the case of insulin, three out of four tyrosine residues were 'free' and the remaining one was 'bound' possibly by a hydrogen bonding, and that one hydroxyl ion was involved in the ionization of this 'bound' residue. In the molecule of lysozyme, one tyrosine residue out of three was found to be strongly hydrogen-bonded, requiring two hydroxyl ions for its ionization. Analysis of catalase molecule showed that out of its total 93 tyrosine residues 26 were in 'bound' states, and that of these 19 were 'strongly bound' and 7 were 'weakly bound'. The

weakly bound residues seems to be those exposed when the native catalase molecule is split by alkali into its three centrifugally homogeneous subunits. The strongly bound residues were found to require two hydroxyl ions for their ionization, which suggested that they may be hydrogen-bonded in the interior or at the surface of the native catalase molecule. The ionization characteristics of various tyrosine residues in the proteins studied were discussed, referring to the data reported by other investigators.

The auther wishes to express his heartfelt thanks to Prof. K. Shibata for his guidance and encouragement during this investigation and to Miss. T. Kurozumi for her kind help in the present study. The author also wishes to thank Prof. H. Tamiya very much for many suggestions in the preparation of this manuscript.

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Studies on Taka-amylase A

VII. Transmaltosidation by Taka-amylase A

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It has been confirmed in our laboratory that crystalline Taka-amylase A is able to hydrolyse phenyl- and *p*-nitrophenyl-α-maltoside to maltose and corresponding phenols and this phenyl-α-maltosidase activity is an intrinsic property of Taka-amylase protein itself (*1*).

In this paper the results of the experiment aiming to examine the nature of transferase activity toward maltosides are reported.

EXPERIMENTAL

Taka-amylase A—Crystalline Taka-amylase A was prepared from "Takadiastase Sankyo" according to the method of Akabori *et al.* (*2*) and recrystallized three times from aqueous acetone.

α-Maltosidase Activity—Maltosidase activity was measured at pH 5.3 (0.5 *M* acetate buffer) and 37°C by determining a reducing power by the method of Fuwa (*3*) and liberated phenol or *p*-nitrophenol by the method of Matsubara *et al.* using phenyl-α-maltoside as substrate (*1*).

Preparation of the α-Maltosides; 1. Alkyl-α-maltosides—Alkyl-α-maltosides could be prepared by warming maltose with an alcoholic solution of hydrochloric acid. Dry hydrogen chloride, was passed into 200 g. of anhydrous alcohol, with ice cooling and exclusion of moisture, until the increase in weight amounted to 10 g. Twenty ml. of this solution was then diluted with 980 g. of alcohol, a 0.1% solution of hydrogen chloride being thus obtained. To this solution 50 g. of finely powdered anhydrous maltose was added, and, with soda-lime tube fitted at the end of the condenser, the mixture was warmed below 50°C for forty-two hours, whereby a clear solution was obtained after the first fifteen minutes. The clear pale yellow solution was cooled to 0°C, and crystallization was induced by scratching. After standing for two days at 0°C the first crop was filtered by suction and washed twice with 100 ml. portions of cold alcohol. The mother liquor and washings were returned to the flask and again warmed for forty-two hours under reflux at 50°C. The liquid was concentrated to 800 ml. and again refrigerated to 0°C, and allowed to stand for 2 days. The second crop of maltoside so obtained was filtered by suction and washed three times with 100 ml. portions of cold alcohol. The mother liquor and washings were combined and concentrated to about 300 ml., refrigerated to 0°C, and again allowed to stand for two days. The resulting mush of crystals was dissolved in twice its weight of absolute ethanol, and, after standing two days at 0°C, the third crop of maltoside was filtered. This was recrystallized from 2.5 parts of ethanol. The product was contaminated by slight traces of maltose and possessed a very faint reducing power towards Fehling's solution. For complete purification it was recrystallized (practically with neither loss of weight nor change in melting point) from five parts of ethanol with the use, if necessary, of decolorizing carbon.

Methyl-α-maltoside, crystallized from ethanol, m.p. 201–202°C, $[\alpha]_D^{16}$ 183° (C=1.0, in water). (Found: C, 43.77; H, 6.95. Calcd. for $C_{13}H_{24}O_{11}$: C, 43.80, H, 6.79 per cent).

*iso*Propyl-α-maltoside, crystallized from ethanol, m.p. 177–179°C, $[\alpha]_D^{16}$ 166° (C=1.0, in water). (Found: C, 46.81; H, 7.32. Calcd. for $C_{15}H_{28}O_{11}$: C, 46.85; H, 7.35 per cent).

*tert*Butyl-α-maltoside, crystallized from ethanol, m.p. 188–190°C, $[\alpha]_D^{16}$ 153° (C=1.0, in water). (Found: C, 48.18; H, 7.62. Calcd. for $C_{16}H_{30}O_{11}$: 48.21; H, 7.59 per cent).

*iso*Amyl-α-maltoside, crystallized from ethanol, m.p. 152–155°C, $[\alpha]_D^{16}$ 143° (C=1.5, in water). (Found: C, 49.52; H, 7.80. Calcd. for $C_{17}H_{32}O_{11}$: C, 49.48; H, 7.82 per cent).

2. Aryl-α-maltosides—Crystallized aryl-maltosides expect the substituted phenyl-α-maltosides were synthesized by the methods of Matsubara (*4*) and Jansen *et al.* using $TiCl_4$ (*5*).

Preparation of phenyl-α-maltoside and its substituted phenyl-α-maltosides were reported in a previous paper (*1*, *4*).

α-Naphthyl-α-maltoside, crystallized from ethanol/ether, m.p. 156–158°C, $[\alpha]_D^{16}$ 192° (C=1.5, in water). (Found: C, 56.41; H, 6.07. Calcd. for $C_{22}H_{28}O_{11}$: C, 56.39; H, 6.03 per cent). The heptaacetyl derivative crystallized from ethanol/ether, m.p. 192–194°C, $[\alpha]_D^{16}$ 185° (C=1.5, in chloroform). (Found: C, 56.71; H, 5.53. Calcd. for $C_{36}H_{42}O_{18}$: C, 56.67; H, 5.56 per cent).

Cyclohexyl-α-maltoside, crystallized from ethanol/ether, m.p. 112–115°C, $[\alpha]_D^{16}$ 211° (C=1.5, in water). (Found: C, 50.85; H, 7.63. Calcd. for $C_{18}H_{32}O_{11}$: C, 50.91; H, 7.60 per cent). The heptaacetyl derivative crystallized from ethanol, m.p. 152–155°C, $[\alpha]_D^{16}$ 202° (C=1.5, in chloroform). (Found: C, 53.42; H, 6.50. Calcd. for $C_{32}H_{46}O_{18}$: C, 53.46; H, 6.46 per cent).

Analytical Methods—Enzymatic transfer reactions were carried out in the reaction mixture of the following composition: 2.0 ml. of 1 *M* phenyl- or *p*-nitrophenyl-α-maltoside, 4.0 ml. of 0.2 *M* acetate buffer of pH 5.3, 2.0 ml. of 5 *M* methanol (ethanol or *n*-butanol) and 2.0 ml. of 10 *μM* Taka-amylase A. Mixture without acceptor contained 2.0 ml. water in place of alcohol. The reaction temperature was 37°C. Under these reaction conditions phenol or *p*-nitrophenol was liberated whereas maltose was partly set free, partly accepted by alcohol forming alkyl-α-maltoside. At varying time intervals 1.0 ml. aliquots were withdrawn, and the liberated phenol or *p*-nitrophenol was determined colorimetrically (*1*) and free maltose reductometrically (*3*).

RESULTS

The extent of maltose transfer from phenyl- or *p*-nitrophenyl-α-maltoside to alcohol was given in Tables I and II. The data listed in the tables indicate that, while in

TABLE I

The Degree of Maltose Transfer to Alcohols by Taka-amylase A Using Phenyl-α-maltoside as Donor

acceptor	reaction time (hours)	substrate cleaved		maltose transferred to alcohol, % of phenyl-α-maltoside cleaved
		phenol liberated %	maltose liberated %	
methanol	1	7.60	6.90	8.29
	9	40.34	37.06	8.13
	28	71.34	65.51	8.17
	50	80.67	73.38	9.04
	120	82.60	75.55	8.52
ethanol	1	8.20	7.08	13.66
	9	41.34	37.61	9.02
	28	74.00	67.33	9.01
	50	83.00	75.58	8.94
	120	83.80	76.76	8.40
n-butanol	1	7.54	6.57	12.87
	9	34.34	30.46	11.70
	28	60.00	52.29	12.85
	50	69.67	59.08	15.20
	120	70.00	59.32	15.27
none	1	7.87	7.88	−0.13
	9	43.47	43.12	0.81
	28	76.00	75.41	0.78
	50	85.00	85.32	−0.37
	120	85.60	86.20	−0.70

Conditions were described in the section of EXPERIMENTAL.

TABLE II

The Degree of Maltose Transfer to Alcohols by Taka-amylase A Using p-Nitrophenyl-α-maltosides as Donor

acceptor	reaction time (hours)	substrate cleaved		maltose transferred to alcohol, % of *p*-nitrophenyl-α-maltoside cleaved
		p-nitrophenol liberated %	maltose liberated %	
methanol	1	11.40	10.35	9.21
	9	60.51	55.06	9.01
	28	64.21	58.26	9.27
	50	68.57	62.37	9.05
	120	71.02	64.62	9.12
ethanol	1	11.80	10.52	10.85
	9	62.01	56.22	9.34
	28	66.60	60.50	9.16
	50	70.51	63.94	9.32
	120	71.93	65.25	9.29
n-butanol	1	11.04	9.86	10.32
	9	51.52	46.19	10.34
	28	54.00	48.26	10.63
	50	57.22	51.12	10.66
	120	59.50	53.42	10.22
none	1	11.82	11.83	−0.08
	9	65.21	64.68	0.81
	28	68.40	67.87	0.78
	50	72.25	72.15	0.14
	120	72.76	72.65	0.15

Conditions were described in the section of EXPERIMENTAL.

the absence of alcohol the enzymatic cleavage of phenyl- or *p*-nitrophenyl-α-maltoside resulted in the liberation of phenol or *p*-nitrophenol and maltose in equimolecular ratio, the presence of alcohol led to the liberation of maltose always smaller in amount than that of phenol or *p*-nitrophenol. This deficit of free maltose could be explained as having been transferred to alcohol. These results indicated that hydrolysis and transfer action proceeded nearly parallel in two substrate maltosides.

As shown in Fig. 1, phenyl-α-maltoside was tested as substrate by first mixing 1 ml. of 2.2% material in 5% methanol with an equal volume of 0.12% Taka-amylase A in 1 ml. of 0.2 *M* acetate buffer, pH 5.3. The mixture was divided into two aliquots. One sample, which served as a control, was immediately placed in a boiling water bath for five minutes. Both samples were then incubated at 37°C for 0.5 to 24 hours, after which the active sample was placed in the boiling water bath for 5 minutes, or deproteinized by adding 0.5 ml. of trichloroacetic acid. Of each aliquot, 0.2 ml. were placed at parallel positions on Toyo No. 51 filter paper and developed for 3 days with the organic phase of a butanol-acetic acid-water mixture (4:1:5) by the ascending technique. After drying, the chromatogram was stained with ammoniacal silver nitrate reagent. The sugar containing products formed by the enzyme action were maltose and methyl-α-maltoside. No glucose was produced from phenyl-α-maltoside.

When p-nitrophenyl-α-maltoside was used as the substrate, formation of methyl-α-maltoside was also chromatographically demonstrated.

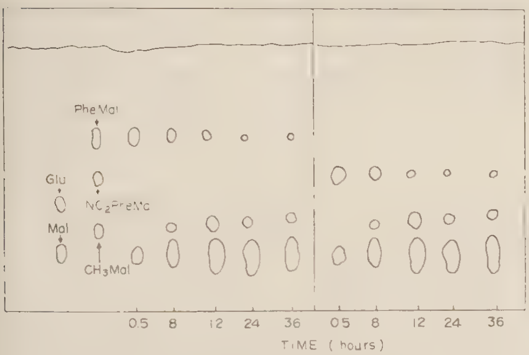

FIG. 1. Chromatogram of the products obtained after incubation of phenyl- and p-nitrophenyl-α-maltosides with crystalline Taka-amylase A in the presence of methanol.

Incubation mixtures contained: 1 ml. of (0.12 mg.) of enzyme solution, 1 ml. of 5 M phenyl (or p-nitrophenyl)-α-maltoside, 1.5 M methanol in 0.2 M acetate buffer, pH 5.3, to make the volume 4 ml. Incubation was made at 37°C, for 0.5, 8, 12 and 24 hours; for deproteinization 0.5 ml. of trichloroacetic acid was used.

Glu, glucose; Mal, maltose; CH_3Mal, methyl-α-maltoside; PheMal, phenyl-α-maltoside; No$_2$ PheMal, p-nitrophenyl-α-maltoside. Ammoniacal silver nitrate solution was used as a spraying reagent for revealing the presence of sugars (*6–7*). Developing reagent: butanol-acetic acid-water (4:1:5), Ascending technique.

Another evidence for the formation of methyl-α-maltoside by the enzymatic transfer of maltose by Taka-amylase A was furnished by the isolation of methyl-α-maltoside and by means of paper chromatography. This experiment was performed with p-cresyl-α-maltoside (*4*) as maltose donor and methanol as α-maltoside acceptor. Such a combination of donor and acceptor was particularly suited for methyl-α-maltoside to accumulate in the reaction mixture, as the rate of hydrolysis by Taka-amylase A of this substrate was considerably large compared with that of the transfer product methyl-α-maltoside. Two and half g. of p-cresyl-α-maltoside was dissolved in 100 ml. 5% methanol, to which 10 mg. Taka-amylase A was added. The whole solution was made to pH 5.3 with acetate buffer. After incubation at 37°C for 10 hours, at the end of which time p-cresyl-α-maltoside had been completely split, the reaction mixture was heated to boiling and the precipitate formed was filtered off. p-Cresol was removed by repeated extraction with ether. The solution was heated again and the clear filtrate was concentrated under reduced pressure. The resulting syrup was dissolved in 10 ml. of hot ethanol and filtered. On keeping the solution in an ice-box crystals deposited. They were filtered, washed with ethanol and recrystallized from absolute ethanol. The product was identified to be methyl-α-maltoside by means of paper chromatography. The R_f values were 0.32 (n-butanol-acetic acid-water, 4:1:5) and 0.56 (70% phenol).

TABLE III

Action of Taka-amylase A on Dilute Solution of Maltose and Methanol (or n-Butanol)

acceptor	reaction time (hours)	free maltose mg.
methanol	1	68.3
	9	68.4
	28	68.2
	50	68.3
	120	68.2
n-butanol	1	68.4
	9	68.3
	28	68.4
	50	68.2
	120	68.3

Reaction mixture was used with the following components: 2.0 ml. of 1 M maltose, 4.0 ml. of 0.2 M acetate buffer of pH 5.3, 2.0 ml. of 5 M methanol (or n-butanol) and 2.0 ml. of 1 mM Taka-amylase A. Incubation temperature wa s37°C.

In order to ascertain whether the formation of methyl-α-maltoside from maltose and methanol had not taken place under the same condition, as Table III illustrated, dilute solution of maltose (0.01 M) and methanol (1.5 M) were incubated with Taka-amylase A.

However, no decrease of free maltose could be ascertained, indicating that the maltoside formation had not taken place.

The effect of various factors for the transfer reaction was studied using methanol as acceptor.

a. Effect of pH—For the purpose of testing maltotransferase individuality of α-maltosidase (Taka-amylase A itself), determination of the optimum pH and a comparison of α-maltosidase with maltotransferase activity were carried out in Taka-amylase A. The result was shown in the following figures.

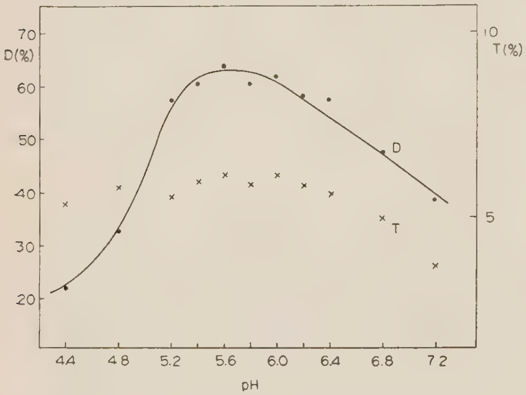

FIG. 2. Effect of pH on the cleavage of phenyl-α-maltoside and the maltose transfer.

Conditions of the reaction : phenyl-α-maltoside 0.5 *M*, methanol 1.5 *M* and enzyme 10 *μM*. Incubated for 8 hours at pH 5.3 (0.2 *M* acetate buffer) and at 37°C.

D represents the percentage of donor split by enzyme as measured by the amount of phenol liberated. If d represents the percentage of maltose liberated, $T=\frac{(D\text{-}d)}{D}\times 100$ will give the percentage of maltose transferred from donor to acceptor.

It can be clearly seen in Fig. 2 that the optimum pH of the cleavage of substrate D lies at about 5.6. The transferase activity was less influenced by pH. The same value of the optimum pH was also obtained in the hydrolysis of *p*-nitrophenyl- and *o*-cresyl-α-maltosides.

b. Effect of Reaction Time—Both donors, phenyl- and *p*-nitrophenyl-α-maltoside, were very rapidly hydrolysed by Taka-amylase A. The degree of cleavage D was affected by reaction time, but the percentage of transfer T was constant under these conditions as shown in Table I and II.

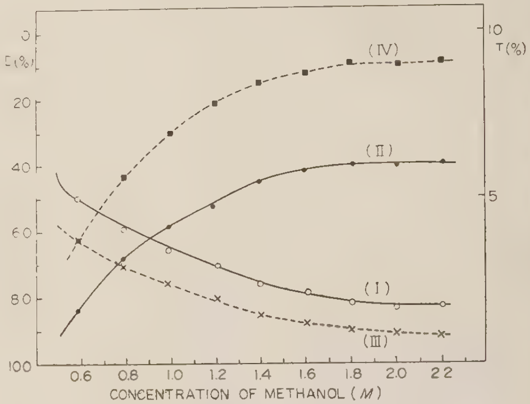

FIG. 3. Effect of the concentration of methanol on the cleavage of phenyl- and *p*-nitrophenyl-α-maltoside and the percentage of maltose transfer.

I. Cleavage D of phenyl-α-maltoside.
II. Transfer T of maltose from phenyl-α-maltoside.
III. Cleavage D of *p*-nitrophenyl-α-maltoside.
IV. Transfer T of maltose from *p*-nitrophenyl-α-maltoside.

D and T were described in Fig. 2. Donors: 0.5 *M* α-maltoside, Acceptor: 1.5 *M* methanol, and Enzyme: 10 *μM*. Incubated for 8 hours (phenyl-α-maltoside), or 5 hours (*p*-nitrophenyl-α-maltoside) at pH 5.3 (0.2 *M* acetate buffer) and at 37°C.

TABLE IV

Effect of the Concentration of Phenyl-α-maltoside as Donor on the Enzymatic Transfer of Maltose to Methanol

molar concentration of donor	0.1	0.5	1.0	1.5
D	25	22	20	17
T	3.8	6.3	8.3	8.5

D and T were described at Fig. 2. Conditions of initials: methanol 1.5 *M* and Taka-amylase A 10 *μM*—4 hours of contact at pH 5.3 (0.2 *M* acetate buffer). Incubation temperature was 37°C.

c. Effect of the Concentration of Acceptor and

Donor—The results illustrated in Fig. 3 indicate that the degree of transfer T to methanol increased continuously with increasing methanol concentration (curves II and IV). However, methanol caused to decrease the cleavage of donor (curves I and III). The data illustrated in Table IV indicates that the percentage of the transfer T increased with increasing donor concentration.

DISCUSSION

With Taka-amylase A an approximate parallelism was found between the activity of hydrolysis of aryl-α-maltosides and that of transfer of maltose residue to alcohols. On the basis of these results, it was concluded that the transfer activity of Taka-amylase A might be attributed to an inherent characteristic action of α-maltosidase itself, namely maltotransferase and α-maltosidase are identical.

In recent years it has been recognized that a large number of hydrolases also catalyse a group transfer from certain donors to acceptors other than water (*8–9*), since hydrolysis almost always accompanies such transferase activity. In view of these results it appears of interest to examine whether or not such transfer action may also be found with other acceptors.

SUMMARY

1. It has been recognized in Taka-amylase A that the enzymatic hydrolysis of aryl-α-maltoside in the presence of various kinds of alcohol is accompanied by the transfer of a part of maltose residue to alcohol, resulting in the formation of corresponding alkyl-α-maltoside.

2. The association of hydrolytic and transfer activities of Taka-amylase A has been considered as suggesting the identity of hydrolase and transferase, where water and alcohol act respectively as the acceptor of maltose residue.

3. The effect of various factors on the transfer reaction has been studied using methanol as acceptor. The results obtained in respect pH, reaction time and donor and acceptor concentrations seem to show that the same enzyme may both hydrolyse the donor into maltose and phenol compound, and catalyse the transfer of maltose of the donor to an alcohol compound.

The author wishes to express his gratitude to Prof. S. Akabori (Institute for Protein Research, Osaka University) for his helpful advice through this investigation, and also to Sankyo Co., Ltd. for their kind supply of "Takadiastase Sankyo".

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Studies on Taka-amylase A

VIII. Influence of Substituents in Phenyl Group on the Enzymatic Hydrolysis of Phenyl-α-Maltoside by Taka-amylase A

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Detailed information on the mode of interaction of an enzyme with its substrate can probably best be obtained by correlating enzyme activity with structural changes in the substrate. The action of enzyme on various glycosides has been extensively studied by Helferich (*1*) and Veibel (*2*).

According to the widely accepted view of Michaelis and Menten (*3*), the action of an enzyme on a substrate falls into two distinct stages, namely, reversible combination to form an enzyme substrate complex and irreversible breakdown of this complex into regenerated enzyme and products; structural changes in the substrate will not necessarily have similar effects on these two stages of the overall reaction and it is therefore desirable to obtain a quantitative measure of both, the first in terms of its Michaelis constant and the second in terms of its velocity constant. The author describes below the determination of both of these constants for taka-amylase A catalysed hydrolyses of phenyl-α-maltoside and its substitution products, together with the determination of the velocity constants for the acid- and alkali-catalysed hydrolyses of the same series of maltosides. In the present work, taka-amylase A was chosen, because the enzyme is readily accessible in crystalline state (*4*). The group of substrates chosen for this work was that of phenyl-α-maltoside (*5–6*), in which structural changes can readily be brought about by the introduction of substituents into benzene ring. The electronic effects of such substituents can be measured quantitatively by means of the substitution constants (δ value) of Hammett (*7–9*).

EXPERIMENTAL

Preparation of the Phenyl-α-maltosides—All of the maltosides were prepared by way of their heptaacetyl derivatives, most of which were prepared by the following procedure (*5*). The phenol (0.3–0.4 mole) was heated with octaacetyl-β-maltose (0.1 mole) and fused zinc chloride (0.5–2.0 g.) at 110–125°C for 1.5 to 2 hours. The cooled product was dissolved in benzene (100 ml.), and the benzene solution was washed with water and then repeatedly with 1 *N* NaOH solution until the washings became colorless. The solution was again washed with water, dried ($CaCl_2$) and the benzene was removed under reduced pressure. The residue was crystallized from suitable solvent, and this crude product was then recrystallized to constant m.p. from a suitable solvent. In certain cases (the nitro compounds), it was found advantageous to add titanic chloride (10 g.) (*10*) in the above reaction mixture and carry out under same condition, but the other synthetic method of *p*-nitrophenyl-α-maltoside was already reported by Matsubara *et al.* (*5*).

The heptaacetylphenyl-α-maltosides were deacetylated by dissolving in anhydrous methanol (200–300 ml. for 25 g.) and adding a freshly prepared solution of sodium methoxide (from sodium, 10–20 mg., and methanol 10 ml.). The mixture was kept at room temperature overnight. The methanol was then removed under reduced pressure, and the residual maltoside was crystallized to constant m.p. using, if possible, more than one solvent. The properties of the maltosides prepared were as follows:

Phenyl-α-maltoside, crystallized from ethanol, m.p. 202–204°C, $[\alpha]_D^{14.5}$ 211° (C=1.3, in water) (*5*). (Found: C, 51.57; H, 6.25. Calcd. for $C_{18}H_{26}O_{11}$: C, 51.65; H, 6.27 per cent).

o-Cresyl-α-maltoside, crystallized from ethanol/ether, m.p. 182–183°C, $[\alpha]_D^{14.5}$ 218° (C=1.0, in water). (Found: C, 52.50; H, 6.45. Calcd. for $C_{19}H_{28}O_{11}$: C, 52.75; H, 6.53 per cent). The heptaacetyl derivative crystallized from ethanol, m.p. 175–176°C, $[\alpha]_D^{18}$

182° (C=1.5, in chloroform). (Found: C, 54.60; H, 5.89. Calcd. for $C_{33}H_{42}O_{18}$: C 54.56 H, 5.83 per cent).

m-Cresyl-α-maltoside, was very hygroscopic and resisted crystallization; the product, dried at 100°C *in vacuo* over P_2O_5, had m.p. 93–98°C, $[\alpha]_D^{17}$ 235° (C=0.5, in water). The heptaacetyl derivative crystallized from ethanol, m.p. 135–140°C, $[\alpha]_D^{18}$ 185° (C=1.5, in chloroform). (Found: C, 54.53; H, 5.84 per cent).

p-Cresyl-α-maltoside, crystallized from ethanol/ether, m.p. 192–194°C, $[\alpha]_D^{18}$ 204° (C=0.5, in water). (Found: C, 53.02; H, 6.49 per cent). The heptaacetyl derivative crystallized from ethanol, m.p. 180–181°C, $[\alpha]_D^{18}$ 201° (C=1.0, in chloroform). (Found: C, 54.55; H, 5.81 per cent).

*o-iso*Propylphenyl-α-maltoside, crystallized from ethanol/petroleum ether, m.p. 163–165°C, $[\alpha]_D^{18}$ 222° (C=1.0, in water). (Found: C, 54.57; H, 7.10. Calcd. for $C_{21}H_{32}O_{11}$: C, 54.75; H, 7.01 per cent). The heptaacetyl derivative crystallized from ethanol/petroleum ether, m.p. 110–111°C, $[\alpha]_D^{18}$ 205° (C=1.0, in chloroform). (Found: C, 55.67; H, 6.08. Calcd. for $C_{35}H_{46}O_{18}$: C, 55.71; H, 6.15 per cent).

*p-iso*Propylphenyl-α-maltoside, crystallized from ethanol/petroleum ether, m.p. 175–176°C, $[\alpha]_D^{14.5}$ 216° (C=1.0, in water). (Found: C, 54.71; H, 6.98 per cent). The heptaacetyl derivative crystallized from ethanol/petroleum ether, m.p. 145–147°C, $[\alpha]_D^{14.5}$ 192° (C=1.0, in chloroform). (Found: C, 55.61; H, 6.21 per cent).

o-tert-Butylphenyl-α-maltoside was very hygroscopic and resisted crystallization; the product, dried at 100°C *in vacuo* over P_2O_5, had m.p. 60–65°C, $[\alpha]_D^{18}$ 232° (C=0.5, in water). The heptaacetyl derivative crystallized from ethanol/petroleum ether, m.p. 212–214°C, $[\alpha]_D^{18}$ 202° (C=1.15, in chloroform). Found: C, 56.26; H, 6.28. Calcd. for $C_{36}H_{48}O_{18}$: C, 56.22; H, 6.30 per cent).

p-tert-Butylphenyl-α-maltoside, crystallized from ethanol, m.p. 170–172°C, $[\alpha]_D^{18}$ 228° (C=0.62, in water). (Found: C, 55.61; H, 7.20. Calcd. for $C_{22}H_{34}O_{11}$: C, 55.66; H, 7.23 per cent). The heptaacetyl derivative crystallized from ethanol/petroleum ether, m.p. 78–80°C, $[\alpha]_D^{18}$ 211° (C=1.32, in chloroform). (Found: C, 56.21; H, 6.26 per cent).

*p-iso*Amylphenyl-α-maltoside, crystallized from ethanol, m.p. 160–163°C, $[\alpha]_D^{18}$ 242° (C=0.65, in water). (Found: C, 56.48; H, 7.47. Calcd. for $C_{23}H_{36}O_{11}$: C, 56.52; H, 7.43 per cent). The heptaacetyl derivative crystallized from ethanol/petroleum ether, m.p. 185–187°C, $[\alpha]_D^{18}$ 213° (C=1.2, in chloroform). (Found: C, 56.80; H, 6.44. Calcd. for $C_{37}H_{50}O_{18}$: C, 56.80; H, 6.44 per cent).

o-Methoxyphenyl-α-maltoside, crystallized from ethanol, m.p. 165–166°C, $[\alpha]_D^{14.5}$ 184° (C=0.1, in water). (Found: C, 50.78; H, 6.27. Calcd. for $C_{19}H_{28}O_{12}$: C, 50.87; H, 6.30 per cent). The heptaacetyl derivative crystallized from ethanol, m.p. 140–141°C, $[\alpha]_D^{14.5}$ 162° (C=0.1, in chloroform). (Found: C, 53.36; H, 5.65. Calcd. for $C_{33}H_{42}O_{19}$: C, 53.38; H, 5.70 per cent).

m-Methoxyphenyl-α-maltoside, crystallized from ethanol, m.p. 172–175°C, $[\alpha]_D^{14.5}$ 193° (C=0.95, in water). (Found: C, 50.79; H, 6.26 per cent). The heptaacetyl derivative crystallized from ethanol, m.p. 163–165°C, $[\alpha]_D^{14.5}$ 165° (C=0.95, in chloroform). (Found: C, 52.90; H, 5.59 per cent).

p-Methoxyphenyl-α-maltoside, crystallized from ethanol, m.p. 187–189°C, $[\alpha]_D^{14.5}$ 201° (C=0.85, in water). (Found: C, 50.80; H, 6.28 per cent). The heptaacetyl derivative crystallized from ethanol, m.p. 206–208°C, $[\alpha]_D^{14.5}$ 195° (C=3.1, in chloroform). (Found: C, 53.31; H, 5.78 per cent).

o-Chlorophenyl-α-maltoside, crystallized from ethanol, m.p. 193–196°C, $[\alpha]_D^{18}$ 165 (C=0.5, in water). (Found: C, 47.68; H, 5.60; Cl, 7.84. Calcd. for $C_{18}H_{25}O_{11}Cl$: C, 47.72; H, 5.57; Cl, 7.83 per cent). The heptaacetyl derivative crystallized from ethanol/petroleum ether, m.p. 195–197°C, $[\alpha]_D^{18}$ 154° (C=2.3, in Chloroform). (Found: C, 51.38; H, 5.23; Cl, 4.68. Calcd. for $C_{32}H_{39}O_{18}Cl$: C, 51.44; H, 5.27; Cl, 4.75 per cent).

p-Chlorophenyl-α-maltoside, crystallized from ethanol, m.p. 205–206°C, $[\alpha]_D^{19}$ 213° (C=1.13, in water). (Found: C, 47.70; H, 5.59; Cl, 7.81 per cent). The heptaacetyl derivative crystallized from ethanol/petroleum ether, m.p. 197–199°C, $[\alpha]_D^{19}$ 177° (C=1.5, in chloroform). (Found: C, 51.43; H, 5.25; Cl, 4.75 per cent).

o-Nitrophenyl-α-maltoside, crystallized from ethanol/ether, m.p. 153–155°C, $[\alpha]_D^{14.5}$ 245° (C=1.2, in water). (Found: C, 46.75; H, 5.48; N, 2.95. Calcd. for $C_{18}H_{25}O_{13}N$: C, 46.63; H, 5.44; N, 3.02 per cent). The heptaacetyl derivative crystallized from ethanol, m.p. 185–187°C, $[\alpha]_D^{14.5}$ 222° (C=1.5, in chloroform). (Found: C, 50.68; H, 5.15; N, 1.84. Calcd. for $C_{32}H_{39}O_{20}N$; C, 50.74; H, 5.19; N, 1.85 per cent).

m-Nitrophenyl-α-maltoside, crystallized from ethanol/petroleum ether, m.p. 132–136°C, $[\alpha]_D^{14.5}$ 261° (C=2.1, in water). (Found: C, 46.78; H, 5.39; N, 2.95 per cent). The heptaacetyl derivative crystallized from ethanol, m.p. 170–171°C, $[\alpha]_D^{14.5}$ 234° (C=1.8, in chloroform). (Found: C, 50.65; H, 5.11; N, 1.87 per cent).

p-Nitrophenyl-α-maltoside, crystallized from ethanol/petroleum ether, m.p. 145–146°C, $[\alpha]_D^{14.5}$ 265° (C=1.0, in water) (*5*). (Found: C, 46.72; H, 5.40; N, 3.04 per cent). The heptaacetyl derivative crystallized from ethanol, m.p. 176–178°C, $[\alpha]_D^{14.5}$ 223° (C=1.2, in chloroform). (Found: C, 50.71; H, 5.21; N, 1.83 per cent) (*5*).

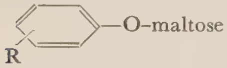
Preparation of the Other α-Maltoside—The alkyl-(methyl, *iso*propyl, *tert*-butyl, and *iso*amyl), α-naphthyl- and cyclohexyl-α-maltosides were all prepared by the procedure of Matsubara (*6*).

Analytical Methods—The enzymatic hydrolyses of all substrates but nitrophenyl-α-maltosides were followed by estimating both the maltose formed by the method of Fuwa (*11*) and the phenol formed by the method of Folin and Ciocalteu (*12*) and Matsubara *et al.* (*5*). The same method was used for following the acid-catalysed hydrolysis of the same maltosides. The alkali-catalysed hydrolyses of all but nitrophenyl-α-maltosides were followed by estimation of the liberated phenols by the method of Folin and Ciocalteu (*12*) and Matsubara *et al.* (*5*); methods based on the estimation of maltose cannot be used, owing to the instability of maltose in alkaline solution. The final color density was measured at 660 mμ.

The nitrophenols were estimated in enzymatic, acid and alkaline hydrolysates by means of their yellow color in alkaline solution. The reaction mixture (1 ml.) was neutrallized if necessary and so diluted with Na_2CO_3

TABLE I

Hydrolyses of Phenyl- and Substituted Phenyl-α-maltosides by Taka-amylase A, Acid, or Alkaline

Substituent 	$10^4 k_0$ (min^{-1})	$10^2 K_m$ (*M*)	$10^{13} k_3$ (min^{-1})	$10^5 k_{acid}$ (min^{-1})	$10^5 k_{alk.}$ (min^{-1})	σ
H	3.25	4.2	16.25	2.30	5.08	0
o-Methyl	15	4.0	72	3.61	2.32	−0.080
m-Methyl	5.45	2.9	20.15	2.81	3.84	−0.069
p-Methyl	4.05	2.5	11.34	4.64	1.93	−0.170
o-*iso*Propyl	26	0.44	32.24	5.32	1.03	—
p-*iso*Propyl	2.21	1.6	5.30	2.51	1.50	−0.151
o-*tert*-Butyl	2.20	1.1	4.27	8.54	0.28	−0.120
p-*tert*-Butyl	1.16	1.45	2.61	4.25	1.35	−0.197
p-*iso*Amyl	1.02	1.1	1.89	6.75	1.45	—
o-Methoxyl	190	2.6	646	17.92	8.45	−0.120
m-Methoxyl	10.5	1.65	25.7	2.43	9.82	+0.115
p-Methoxyl	6.05	1.8	10.85	4.84	1.89	−0.268
o-Chloro	345	0.68	510.6	3.63	460	+0.325
p-Chloro	22.8	0.42	27.81	1.72	17.92	+0.227
o-Nitro	1,250	0.93	2,162.5	3.26	$10^4>$	+0.650
m-Nitro	185	0.68	273.8	0.78	295	+0.710
p-Nitro	195	0.62	276.9	0.59	$10^3>$	+0.778 +1.270

k_0=Overall velocity constant, min^{-1}.

K_m=Michaelis constant for hydrolysis by take-amylase A at 37°C in 0.08 *M* acetate buffer, pH 5.3; *M*.

k_3=First-order velocity constant for decomposition of enzyme substrate complex under above conditions; min^{-1}.

k_{acid}=First-order velocity constant for hydrolysis at 60°C in 0.05 *M* solution in 0.1 *N* HCl; min^{-1}.

$k_{alk.}$=First-order velocity constant for hydrolysis at 60°C in 0.01 *M* solution in 1 *N* NaOH; min^{-1}.

σ=Substituent constant. (Values for *m*- and *p*-substituents from Hammett (*7–9*); for substituents, slightly modified in the light of more recent work, from Mamalis and Rydon (*13*)).

solution as to give a final Na_2CO_3 concentration of 0.5 *N*. The yellow color was measured at 400 mμ.

Acid and Alkaline Hydrolyses—The hydrolysis was carried out in flask in the water thermostat at 60°C. For the acid hydrolysis the maltosides were taken in 0.05 *M* concentration in 0.1 *N* HCl; for the alkaline hydrolysis 0.01 *M* solutions in 1 *N* NaOH were used. Samples were withdrawn at suitable times, neutrallized, and then analysed by the method described above. The first-order velocity constants, k_{acid} and $K_{alk.}$ were evaluated in the usual manner from the satisfactorily linear log plots.

Enzymatic Hydrolysis—The hydrolysis was carried out in 25 ml. flask into which was introduced suitable volumes of acetate buffer, pH 5.3* (optimum conditions for enzymatic hydrolysis), maltoside solution, and enzyme solution to give the total volume of 3 ml., with a buffer concentration of 0.08 *M* and the final concentration of maltoside of 0.01 *M* and enzyme of 1.0 μM; the enzyme solution was added last, after preheating the other components in a thermostat (at 37°C) for 10 minutes. At suitable intervals, 1 ml. samples were withdrawn for analysis; in all cases a blank determination was made using the same concentration of enzyme and of substrate in 0.08 *M* acetate buffer, pH 5.3; some of the substrates (*m*-cresyl and *o-tert*-butylphenyl-α-maltosides) contained small amounts of reducing impurity for which requisite correction was made.

The overall velocity constant, k_0 was calculated in the usual way from the slope of the linear plot (drawn by the method of least squares) of the decadic logarithm of the concentration of unhydrolysed substrate against the time in minutes.

For the determination of K_m and k_3 two sets of three such determinations of k_0, using different enzyme concentrations, were carried out with each substrate. The data for the enzyme, acid and alkaline hydrolyses of phenyl-α-maltoside and the other maltosides are given in Table I.

RESULTS

The results of the experiments on enzymatic hydrolysis are expressed in terms of the equilibrium constant, k_1/k_2, for the formation of the enzyme substrate complex and of the first-order velocity constant, k_3, for the decomposition of this complex according to the well-known Michaelis Menten representation:

$$E+S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E+P$$

It is more usual to use Michaelis constant, K_m, which is the dissociation constant for the breakdown of the enzyme substrate complex into its original components. Veibel and Erikson (*14*) and Veibel and Lillelund (*15*) have showed that, under conditions such that the influence of the products on the velocity of the reaction is negligible, k_3 is calculated using the equation (1),

$$k_3 = \frac{k_0(K_m + S_0)}{e_0} \qquad (1)$$

where S_0 and e_0 are the initial molar concentrations of substrate and enzyme, respectively. The value of e_0, required for substitution in the above-mentioned equation for the evaluation of k_3, can be readily determined since the molecular weight of the enzyme is known. On rearrangement, (1) takes the form (2),

$$\frac{1}{k_0} = \frac{1}{k_3 e_0}(K_m + S_0) \qquad (2)$$

which is the equation for the linear plot of $1/k_0$ against S_0; clearly k_3 could be determined from the slope of this line but, in this paper, the arithmetical method was used. The values of k_0, K_m and k_3 and of the first-order velocity constants, k_{acid} and $k_{alk.}$ for the acid and alkaline hydrolyses of the maltosides investigated were given in Table I.

DISCUSSION

The logarithms of the overall velocity constants, k_0, are plotted against the substituent constants, σ*, in Fig. 1. The plot clearly shows that the reaction is facilitated by the presence of electron-attracting substituents in phenyl group, and that especially the marked facilitation of enzymatic hydrolysis by the *o*-substituents with the exception of *o*-*tert*-butyl compound is very pronounced. The reaction constant, ρ, was calculated to be $+2.00$ from the slope of Fig. 1. In Fig. 2, the logarithms of Michaelis constants are plotted against σ. With the exception of *p*-chloro compound, the fit is about as good as is usually observed in such correlations. The plot clearly shows that the formation of the enzyme substrate complex is facilitated by the presence of electron-attracting substituents in phenyl group. The reaction constant, ρ, was calculated to be -0.45 from the slope of Fig. 2.

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* The author studied the effect of pH on the hydrolyses of a number of maltosides with taka-amylase A and found that the overall velocity constant was almost unaffected by change of pH over the range 5.3～6.0. The author therefore adopted pH 5.3 for the work.

* In order to systematise the effect of substituents in benzene ring, Hammett had suggested the following equation, $\log(k/k_0)=\sigma\rho$; k and k_0 are rate or equilibrium constants for reactions of the substituted and the unsubstituted compounds, and also σ is a substituent constant, dependent upon the substituent, whose value is independent of the reaction and ρ is a reaction constant, dependent upon the reaction, the medium and the temperature but independent of the substituent.

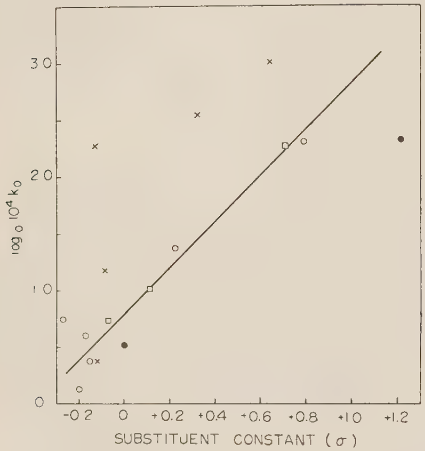

FIG. 1. Correlation of k_0 with structure. ●, Phenyl-α-maltoside; ○, *o*-Substituted phenyl-α-maltosides; □, *m*-Substituted phenyl-α-maltosides; ×, *p*-Substituted phenyl-α-maltosides. The line was drawn by the method of least squares, neglecting the points for the *o*-substituted compounds.

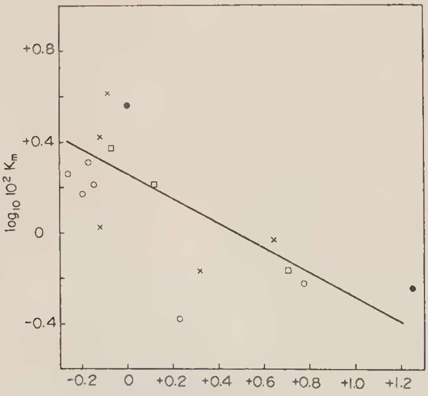

FIG. 2. Correlation of K_m with structure. For key, see Fig. 1. The lines was drawn by the method of least squares, neglecting the points for the *o*-substituted compounds.

The fact that the points for the *o*-substituents fall satisfactorily on the line indicates that steric effects play a negligible part in the interaction; it follows that this interaction must take place in a part of the molecule remote from phenyl group, namely in maltose moiety. The most likely form of such interaction subject to polar influences is hydrogen bonding and the facilitating influence of electron-attracting substituents indicates that this must involve hydroxyl hydrogens of maltose residue and hydrogen-accepting groups in the enzyme molecule; the alternative possibility of hydrogen bonding between oxygens of maltose residue and hydrogens of the enzyme is excluded, since this would be hindered by electron-attracting substituents in phenyl groups. The observations of Helferich and Lang (*16*), of Pigman and Richtmyer (*17*) and of Nath and Rydon (*18*), that certain *o*-methylated glucosides are not hydrolysed by emulsin, are in accordance with the suggested mechanism.

The marked facilitation of enzyme substrate combination by the bulky alkyl groups, *o*- and *p*-*iso*-propyl, *o*- and *p*-*tert*-butyl, and *p*-*iso*amyl, in spite of their electron-repelling properties, suggests that there is some other type of interaction, probably by means of van der Waals forces, involving aryl group. From the result in the present study and the study on the specificity of taka-amylase A for alkyl-α-maltoside (*6*), it is suggested that the phenyl group in α-maltosides is necessary for the enzyme action, and also the suggestion that it is attached by van der Waals forces is in accord with the observations of Veibel and Lillelund (*19*) that the equilibrium constant for combination of emulsin with alkyl β-glucosides increases with increasing size of alkyl group.

The logarithms of the first-order velocity constants, $k_{alk.}$ and k_{acid}, for the alkali- and acid-catalysed hydrolyses are plotted against σ in Figs. 3 and 4. The effect of substitution on the two reactions is that expected on general ground, the acid-catalysed reaction, which depends on attack by the electrophilic proton, being facilitated by electron-repelling substituents, and the alkali-catalysed reaction, in which the attack is by the nucleophilic hydroxyl ion, being facilitated by electron-attracting groups. The reaction constants, ρ for the two reactions, +2.51 and −0.25, respectively. Both reactions show facilitation by *o*-nitro, *o*-chloro and *o*-methoxyl groups. Facilitation of alkaline hydrolysis by *o*-nitro group is very pronounced and has been noted by previous workers; Nath and Rydon (*18*) and Montgomery, Richtmyer and Hudson (*20–21*) and Snyder and Link (*22*). It is readily explicable on the basis of hydrogen bonding with two 2-hydroxyl groups of maltose residue, which leads to enhanced electrophilic reactivity of the relevant carbon-1 atom in maltose residue, and the weaker facilitation by *o*-chloro and *o*-methoxyl groups may have a similar explanation. The effect of these three substituents on the acid-catalysed hydrolysis is not readily explicable. The bulky *iso*propyl, *tert*-butyl and *iso*amyl groups, when present as *o*-substituents, cause inhibition of alkaline hydrolysis, no doubt owing to conventional steric hindrance.

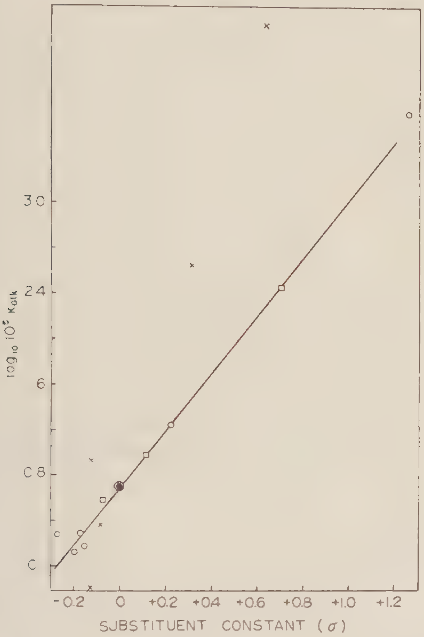

FIG. 3. Correlation of $k_{alk.}$ with structure. For key, see Fig. 1. The line was drawn by the method of least squares, neglecting the points for the *o*-substituted compounds.

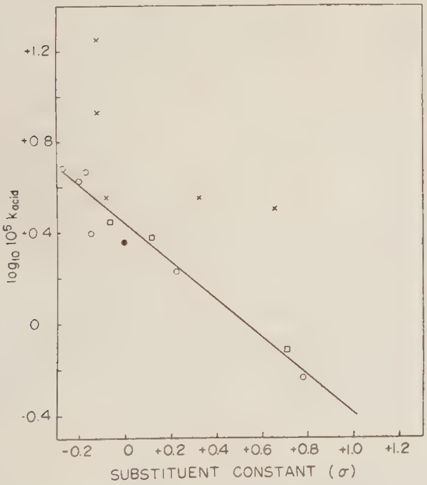

FIG. 4. Correlation of k_{acid} with structure. For key, see Fig. 1. The line was drawn by the method of least squares, neglecting the points for the *o*-substituted compounds.

Fig. 5 shows log k_3 plotted against σ. It

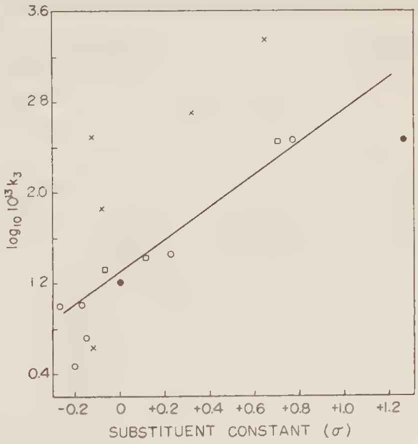

FIG. 5. Correlation of k_3 with structure. For key, see Fig. 1. The line was drawn by the method of least squares, neglecting the points for the *o*-substituted compounds.

is at once apparent that the decomposition of the enzyme substrate complex, although it occurs in acid solution, resembles alkaline, rather than acid, hydrolysis in being facilitated by electron-attracting substituents. Clearly, the enzyme substrate complex decomposes under the attack of some nucleophilic reagents; whether this is free hydroxyl ion, adsorbed hydroxyl ion or hydroxyl ion arising from hydrated amino-groups, is a question which cannot be answered on the basis of the available evidence. It is also clear from Fig. 5 that marked steric influences are superimposed on the more usual electronic influences. The points for the *p*-substituted compounds fall reasonably well on a straight line passing through the point for the unsubstituted compound, with the exception of those for *p-iso*-propyl, *p-tert*-butyl and *p-iso*amyl compounds; the sluggish decomposition of these three complexes is very probably due to the strength of the van der Waals component of the forces holding them together, and it may well be that it is the enzyme product complex which is unusually stable in these causes. The *m*-substituted compounds also fall well on a straight line which is, however, considerably displaced towards high k_3 values from the point for the unsubstituted compound. The *o*-substituted compounds, again with the exception of *tert*-butyl compound, which, like their *p*-isomerides, may owe their stability to strong van der Waals interaction, and also show very markedly enhanced instability; the ratio of the values of k_3 for the *o*- and *p*-isomerides are summarized in Table II.

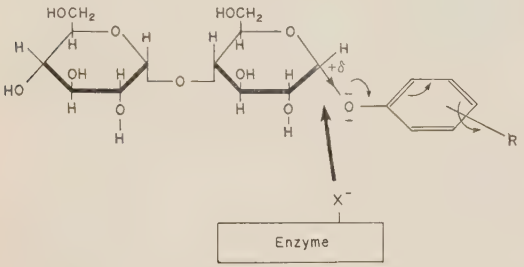

FIG. 6. Possible mode of attachment of substituted phenyl-α-maltoside to taka-amylase A. (Maltose ring lines in the plane of the paper, benzene ring at right angles to it.)

TABLE II

The Ratio of the Values of k_3 for the o- and p-Substituted Phenyl-α-maltosides

Substituent	*o*-substituted	*p*-substituted	$\frac{k_3 \text{ for } o\text{-}}{k_3 \text{ for } p\text{-}}$
Methyl	72	11.34	6.35
*iso*Propyl	32.24	5.30	6.15
tert-Butyl	4.27	2.61	1.63
Methoxyl	646	10.85	59.5
Chloro	510	27.81	18.4
Nitro	2,162.5	276.9	7.85

k_3=First-order velocity constant for decomposition of enzyme substrate complex by taka-amylase A.

Clearly the effect described above as well as in the *m*-series, is due to the electronic properties, rather than the size, of the substituents.

The degree of hydrolyses of the other substituted α-maltosides but the substituted phenyl-α-maltosides is summarized in Table III.

TABLE III

Hydrolyses of the other Substituted α-Maltosides by Taka-amylase A

Substituted α-maltoside	the degree of the substrate hydrolysed %
Methyl	2.1
*iso*Propyl	2.7
tert-Butyl	2.7
*iso*Amyl	3.1
α-Naphthyl	5.0
Cyclohexyl	0

Incubation time was 8 hours at 37°C and pH 5.3 (0.2 *M* acetate buffer). The degree of hydrolysis was followed by estimating maltose formed using a reducing power by the method of Fuwa (*11*).

The facts and discussions described above lead the author to the following inference; the results have been correlated with the electronic properties of the substituents, as measured by their substituent constants, and the substituents in the substrate molecule make it easy that O-bond between maltose residue and phenol group is attacked, and that the key carbon-1 atom of maltose residue is become more accessible to attack by the nucleophilic entity involved in the enzyme molecule; the stereochemical specificity of taka-amylase A renders it likely that at least maltose residue is involved in the attachment of the substrate to the enzyme and the representation in Fig. 6 is advanced as at least a plausible working hypothesis.

SUMMARY

1. The hydrolyses by taka-amylase A of phenyl-α-maltoside and some of its substitution compounds have been studied; Michaelis constants for the dissociation of the enzyme substrate complex and the first-order velocity constants for their decomposition have been estimated.

2. First-order velocity constants for the alkaline and acid hydrolyses of most of maltosides have also been determined.

3. The results have been correlated with the electronic properties of the substituents, as measured by their substituent constants; acid hydrolysis is facilitated by electron-repelling substituents but alkaline hydrolysis and the formation and breakdown of the enzyme substrate complex are all facilitated by electron-attracting substituents.

4. The degree of hydrolysis by taka-amylase A of the other substituted α-maltosides has been described.

5. A mechanism is suggested for the combination and for the decomposition of taka-amylase A with aryl-α-maltosides.

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Studies on Acetic Acid Bacteria

IV. Purification and Properties of a New Type of Alcohol Dehydrogenase, Alcohol-Cytochrome-553 Reductase*

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(Received for publication, October 12, 1960)

Tanenbaum (*1*) suggested that *Acetobacter peroxydans* might contain an alcohol dehydrogenase and an aldehyde dehydrogenase and that both might be TPN-dependent. In previous communications (*2–4*), the author has described two different aldehyde dehydrogenases which were isolated from the acetic acid bacteria used in this vinegar industrial factory. These two dehydrogenases have been highly purified, and it has been confirmed that one is dependent on TPN but not on DPN and the other is coenzyme-independent. They have been found to differ from each other in many points. It was noticed that an extract from the micro-organism had ethanol-ferricyanide reducing activity and cytochrome-like α-absorption band at 553 mμ.

The present paper describes the purification of the alcohol-dye reducing enzyme and on its general properties. The highly purified enzyme has a cytochrome c-like absorption spectrum, and is reduced by the addition of alcohol both in the presence and absence of coenzymes.

MATERIALS AND METHODS

The strain of *Acetobacter* sp. used and the growth conditions were as described previously (*2–4*).

Acetone powder was made from the cells with the conventional method and could be stored for at least one month in the cold (3–4°C) in a dry state without noticeable loss of ethanol-ferricyanide reducing activity.

Reduction of oxidation-reduction dyes and coenzymes in the presence of alcohols was assayed b measuring the decrease in optical density at appropri ate wavelengths using a system containing the follow ing components: 0.1 ml. of enzyme solution, 3.0 ml of 0.04 M citrate buffer, pH 3.8, (except when other wise indicated), 0.1 ml. of 0.2 M substrate, 0.1 ml. o 0.02 M oxidation-reduction dye or 6.0 mM coenzym and water to a total volume of 3.4 ml. Reaction were started by adding the enzyme, and the optica density was followed at room temperature (24°C) i a Shimadzu photoelectric spectrophotometer, type QB-50, using cuvettes of one-cm. optical path. The enzyme activity was calculated from the initial linea reaction rate, and expressed as $-\Delta^{\text{Ferri.}}_{400\text{m}\mu}$/minute fo ferricyanide-reduction. The protein content of the samples was expressed as the optical density at 280 mμ; $\text{E}_{280\text{m}\mu}$. Specific activity was expressed in units per $\text{E}_{280\text{m}\mu}$; $-\Delta\text{E}^{\text{Ferri.}}_{400\text{m}\mu}/\text{E}_{280\text{m}\mu}$/minute for ferricyanide-reduction. The TPN-dependent and coenzyme-independent aldehyde dehydrogenase activities were measured as described in the previous reports (*3*, *4*). In some experiments, reduction of oxidation-reduction dyes was studied anaerobically at 30°C using Thunberg tubes and the time required to decolorize the dyes was measured in minutes.

The concentration of the hemeprotein was expressed as the optical density at 553 mμ of the dithionite-reduced form; $\text{E}^{\text{red.}}_{553\text{m}\mu}$. The specific hemeprotein content was expressed as; $\text{E}^{\text{red.}}_{553\text{m}\mu}/\text{E}_{280\text{m}\mu}$.

Zone electrophoresis on a vertical starch column was carried out by the method of Horio *et al.* (*5*)

Crystalline cytochrome c was prepared from bovine heart muscle by the method of Bodo (*6*) using the slight modification of Yamanaka *et al.* (*7*). Aluminium oxide for chromatography was purchased commercially from Wako Pure Chemical Industrial Co., Ltd., Osaka. The oxide was reactivated by acid and alkali treatment (*5*).

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[*] This work was done in the laboratory of Prof. K. Okunuki, Department of Biology, Faculty of Science, University of Osaka, Osaka.

EXPERIMENTALS AND RESULTS

Purification of the Alcohol-Ferricyanide Reducing Enzyme—The alcohol-ferricyanide reducing enzyme was extracted from acetone-dried cells of *Acetobacter* sp. at various pH's using three kinds of buffer. Seven g. of acetone-dried cells were suspended in 100 ml. of each buffer solution (0.1 *M*) and stirred for 10 minutes in a homo-mixer (15,000 r.p.m.) at room temperature (24°C). The mixture was then centrifuged (10,000 × *g*, 10 minutes). As with the coenzyme-independent aldehyde dehydrogenase (*Acetobacter*) (*4*), if the mixing time was shorter than 10 minutes, little enzyme was extracted. The ethanol-ferricyanide reducing activity of the resulting supernatants was compared. Of the buffers tested, citrate-phosphate buffer was the most effective for extraction (see Fig. 6), and the optimal pH was approximately 3 regardless with the type of buffer used (Fig. 1). In citrate-phosphate buffer, less than one-third as much enzyme could be extractable at pH 4.0 as at pH 3.0, and very little enzyme could be extracted at pH 2.0. The extract obtained using citrate-phosphate buffer, pH 3.0, contained much TPN-dependent aldehyde dehydrogenase (*3*) and coenzyme-independent aldehyde dehydrogenase (*4*), and had a cytochrome-like absorption band at 553 mμ.

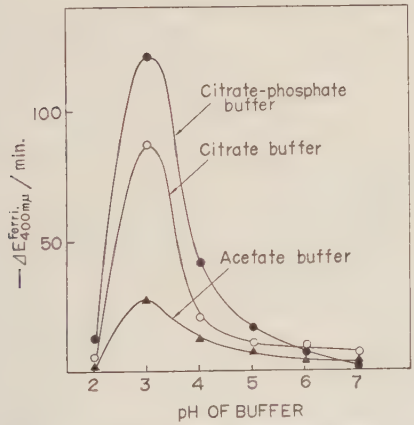


Fig. 1. Effect of pH on extraction of ethanol-ferricyanide reducing enzyme.

To purify the alcohol-ferricyanide reducing enzyme, acetone-dried cells (75 g.) were extracted with 0.1 *M* citrate-phosphate buffer, pH 3.0, under the above conditions. A first and second extraction were made with 1,500 ml. and 750 ml. of buffer, respectively. The second extract had less than one-third as much ethanol-ferricyanide reducing activity as the first extract, while the specific activity of the latter was one-half of the former. The two extracts were combined. The yellowish brown mixture (1,880 ml.) was passed through an aluminium oxide column (15 × 5 cm.) which had been equilibrated with 0.1 *M* citrate-phosphate buffer, pH 3.0. The alcohol-ferricyanide reducing enzyme, the coenzyme-independent aldehyde dehydrogenase, the TPN-dependent aldehyde dehydrogenase and the hemeprotein showing an absorption band at 553 mμ were completely absorbed on the column. The column was then eluted with 0.5 *M* citrate-buffer, pH 5.0, and 5 ml. aliquots were collected (Fig. 2).

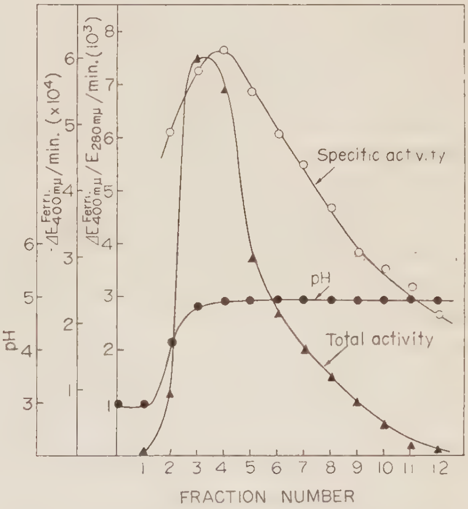


Fig. 2. Chromatography of alcohol-ferricyanide reducing enzyme on pH 3.0-aluminium oxide column.

The best fractions (Fractions No. 2–10, in Fig. 2) were combined.

When the resulting mixture was fractionated at pH 7.0 with ammonium sulfate, 91 per cent of the alcohol-ferricyanide reducing enzyme was precipitated between 40 and 60 per cent saturation of ammonium sulfate, and

TABLE I

Ammonium Sulfate Fractionation of Alcohol-Ferricyanide Reducing Enzyme, Coenzyme-Independent Aldehyde Dehydrogenase and TPN-Dependent Aldehyde Dehydrogenase

Conc. of ammonium sulfate (per cent of saturation)	Color	Total $E_{280m\mu}$	Ethanol-ferricyanide reducing enzyme (units)	Coenzyme-independent aldehyde dehydrogenase (units)	TPN-dependent aldehyde dehydrogenase (units)
40–50	Red	695	5150	1045	0
50–60	Yellowish red	544	4890	7560	27
60–70	Colorless	324	646	2261	193
70–85	Colorless	238	333	333	357

87 per cent of the coenzyme-independent aldehyde dehydrogenase between 50 and 60 per cent saturation of the salt. Seventy-nine per cent of the TPN-dependent aldehyde dehydrogenase precipitated between 60 and 85 per cent saturation of the salt (Table I).

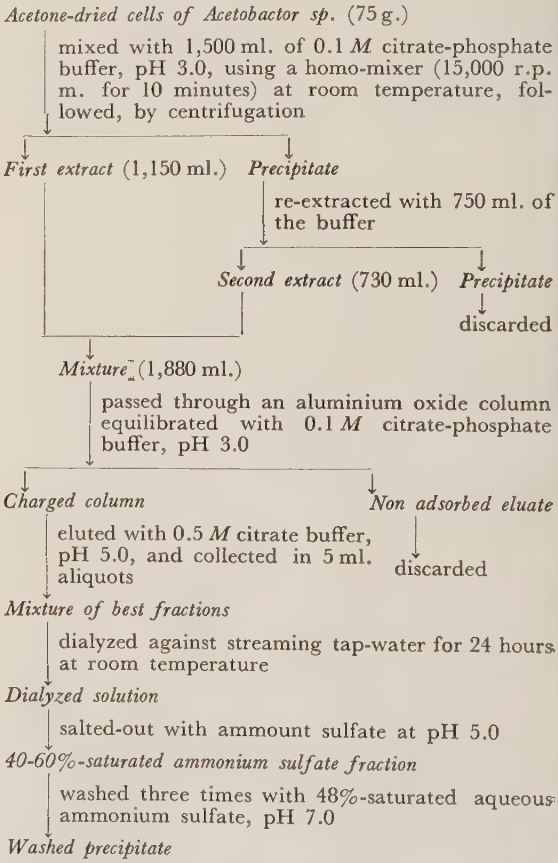
The combined eluate from the pH 3.0-aluminium oxide column was dialyzed at 10–15°C for 24 hours against streaming tap-water. During the dialysis, the TPN-dependent aldehyde dehydrogenase was completely inactivated, while the alcohol-ferricyanide reducing enzyme and the coenzyme-independent aldehyde dehydrogenase only slightly.

The dialyzed solution was then fractionated at pH 5.0 between 40 and 60 per cent saturation with ammonium sulfate. The precipitate which appeared was collected and was washed with 48 per cent saturated ammonium sulfate, pH 7.0, at approximately 10°C, until the coenzyme-independent aldehyde dehydrogenase presenting in the precipitate had been removed. Three washings with approximately ten volumes of aqueous salt solution were satisfactory for this purpose. The resulting alcohol-ferricyanide reducing enzyme solution had 109-fold greater purity than the crude extract and there was a yield of 40 per cent.

The purification procedure is shown in Scheme I, and the purity and the yield achieved at each step are given in Table II. Experiments were usually carried out with enzyme at the final step of purification.

SCHEME I

Purification of Alcohol-Ferricyanide Reducing Enzyme



Acetone-dried cells of Acetobactor sp. (75 g.)

mixed with 1,500 ml. of 0.1 *M* citrate-phosphate buffer, pH 3.0, using a homo-mixer (15,000 r.p.m. for 10 minutes) at room temperature, followed, by centrifugation

First extract (1,150 ml.)

Precipitate

re-extracted with 750 ml. of the buffer

Second extract (730 ml.)

Precipitate

discarded

Mixture (1,880 ml.)

passed through an aluminium oxide column equilibrated with 0.1 *M* citrate-phosphate buffer, pH 3.0

Charged column

Non adsorbed eluate

eluted with 0.5 *M* citrate buffer, pH 5.0, and collected in 5 ml. aliquots

discarded

Mixture of best fractions

dialyzed against streaming tap-water for 24 hours at room temperature

Dialyzed solution

salted-out with ammount sulfate at pH 5.0

40-60%-saturated ammonium sulfate fraction

washed three times with 48%-saturated aqueous ammonium sulfate, pH 7.0

Washed precipitate

Relation between the Ethanol-Ferricyanide Reducing Activity and the Cytochrome-553 Content—During the course of purification of the alcohol-ferricyanide reducing enzyme, there was

TABLE II

Purification of Alcohol-Ferricyanide Reducing Enzyme

Purification step	Enzyme units with ethanol $(-E^{\mathrm{Ferri.}}_{400\mathrm{m}\mu}/\mathrm{min.})$	Specific activity $(-E^{\mathrm{Ferri.}}_{400\mathrm{m}\mu}/E_{280\mathrm{m}\mu}/\mathrm{min.})$	Yield (per cent)	Purity
Mixture of 1st and 2nd extracts	1,890,000	320	(100)	(1)
Eluate from pH 3-aluminium oxide column	1,650,000	4,800	87	15
After dialysis	1,640,000	7,800	87	24
40–60 per cent saturated ammonium sulfate fraction	1,270,000	15,000	67	47
Preparation after washing three times with 48 per cent saturated aqueous ammonium salfate pH 7.0	750,000	35,000	40	109

noticed an apparent relationship between the enzyme activity and the hemeprotein content of the sample. This suggested that the cytochrome c-like hemeprotein itself (cytochrome-553) might be the enzyme. However, until about the middle of the purification procedure, it was difficult to measure the hemeprotein content accurately because of the contamination by many other coloured substances. Therefore, the eluate from the pH 3.0-aluminium oxide column was treated at pH 5.0 with 60 per cent saturated ammonium sulfate. The resulting precipitate was used for the study of the relation between the ethanol-ferricyanide reducing activity and the hemeprotein content. The sample was dialyzed against 0.25 *M* citrate buffer of pH 5.0, and then passed through an aluminium oxide column (15×5 cm.) which had been equilibrated with 0.25 *M* citrate buffer, pH 5.0. The enzyme which was adsorbed on the column was chromatographed with the same buffer. The most active fraction was estimated to have a ratio of specific activity to specific hemeprotein content of 1.6×10^5 (Table III). Some of the material which had been dialyzed against citrate buffer was redialyzed against acetate buffer ($\Gamma/2=0.10$, pH 4.5). Zone electrophoresis was carried out with this solution at 3–4°C on a vertical starch column (30×3 cm.), using the same buffer as an overlying solution. The enzyme migrated to the cathode, indicating that its isoelectric point was higher than pH 4.5. After 24-hours electrophoresis (20 mA, 350 400 V), the column was eluted with the same buffer and fractionated. The most active fraction had a specific activity per hemeprotein content ratio of 1.5×10^5. The enzyme at the final step of purification had a ratio of 1.6×10^5. Thus the ratios obtained with samples purified in different ways were constant. Therefore, it seems likely that the

TABLE III

Relation between Ethanol-Ferricyanide Reducing Activity and Cytochrome-553 Content during Purification

Purification procedure	Specific activity $(-\Delta E^{\mathrm{Ferri.}}_{400\mathrm{m}\mu}/E_{280\mathrm{m}\mu}/\mathrm{min.})$	$E^{\mathrm{red.}}_{553\mathrm{m}\mu}/E_{280\mathrm{m}\mu}$	$-\Delta E^{\mathrm{Ferri.}}_{400\mathrm{m}\mu}/E^{\mathrm{red.}}_{553\mathrm{m}\mu}/\mathrm{min.}$
After chromatography on aluminium oxide column	8,000	0.049	1.6×10^5
After zone-electrophresis	16,000	0.120	1.5×10^5
Enzyme at final stage of purification	35,000	0.220	1.6×10^5

hemeprotein itself has the alcohol-ferricyanide reducing activity.

General Properties of the Ethanol-Ferricyanide Reducing Enzyme

Absorption Spectrum—The alcohol-ferricyanide reducing enzyme at the final step of the purification had a cytochrome c-like absorption spectrum. The hemeprotein was still 30–50 per cent reduced at the final purification step, indicating that the hemeprotein was not very autoxidizable. In its oxidized form the hemeprotein was immediately reduced when ethanol was added to the enzyme solution, and in its reduced form was oxidized by the addition of ferricyanide (Fig. 3). The hemeprotein could be reduced to the same extent by ethanol as by dithionite. Its oxidized form has the absorption maxima at 530 mμ (diffuse), 412 mμ, 355 mμ and 279 mμ, and in the ethanol reduced form at 553 mμ, 522 mμ, 417 mμ, 315 mμ and 279 mμ. Furthermore, the reduced form showed shoulders at 345 mμ and 290 mμ, and the oxidized form a shoulder at at 290 mμ. These absorption spectra were fairly similar to those of c-type cytochrome, but the ratio of the extinction of the reduced α- and β-absorption peaks was nearly unit and a sharp δ-absorption peak of reduced c-type cytochrome was at 315 mμ in the reduced form. A value of the extinction coefficient ($E^{\text{red.}}_{553\text{m}\mu}$) was 0.225×10^5 (cm^2./g. atom Fe).

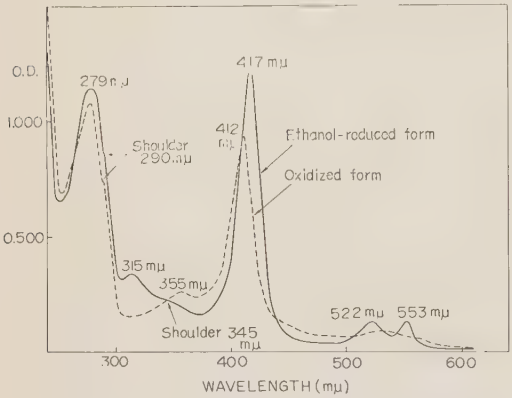


Fig. 3. Absorption spectra of alcohol-ferricyanide reducing enzyme preparation.

Ultracentrifugal Analysis—The alcohol-ferricyanide reducing enzyme preparation dissolved in 0.05 *M* acetate buffer of pH 5.0 was subjected to the measurements of purity and sedimentation constant using a Hitachi analytical ultracentrifuge, UCA-1. The typical sedimentation pattern is shown in Fig. 4.

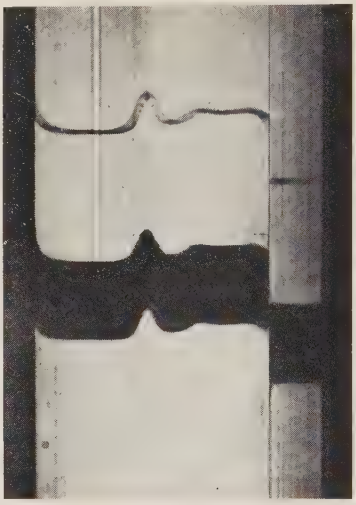


Fig. 4. Sedimentation pattern of a 0.5 per cent solution of alcohol-ferricyanide reducing enzyme preparation in 0.05 *M* acetate buffer, pH 5.0.

In this experiment, average rotor temperature was 18.4°C. The picture was taken 19 minutes after reaching full speed (59,780 r.p.m.).

As seen in sedimentation pattern it was observed that the red color sedimented with the sedimentating boundary and supernatant fraction became completely colorless, and that the main peak was present, together with some polydisperse material of low molecular weight which produced an ill-defined peak. By the conventional area-method, it was calculated that the main peak contained 60–70 per cent of the total sedimentable molecule present in the sample. The $S_{20,w}$ of main peak due to hemeprotein was calculated to be 10.3×10^{-13} cm./sec.. When the partial specific volume (ν) and the frictional ratio (f/f_0) of the hemeprotein were taken as 0.73 ml./g. and 1.25, respectively, the molecular weight was calculated from the $S_{20,w}$ to be approximately 250,000. The values for ν and f/f_0 were chosen with reference to those of catalase which had a similar $S_{20,w}$ (*8*, *9*).

Effect of pH and Heat on Enzyme Activity and Stability—The alcohol-ferricyanide reducing enzyme differed in activity and pH optimum in different buffers (Fig. 5). Of the three buffers tested, use of citrate buffer resulted in the best enzyme activity. The pH optimum was pH 3.8 in citrate buffer and citrate-phosphate buffer, and pH 4.0 in acetate buffer.

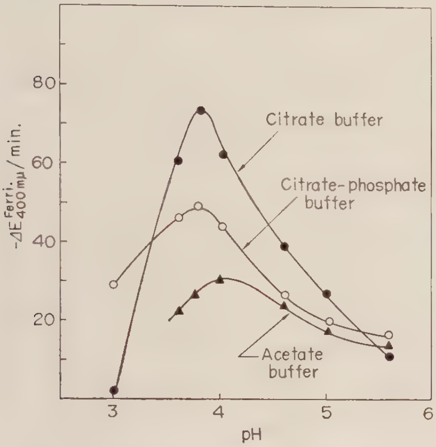


Fig. 5. pH-activity curves of alcohol-ferricyanide reducing enzyme.

In the presence of citrate buffer, the enzyme was fairly stable over the broad pH-range of pH 5.0 to 7.0 (Fig. 6). However, in the absence of this buffer it was only stable at pH 7.0. The enzyme was comparatively stable below 45°C, but not at higher temperatures.

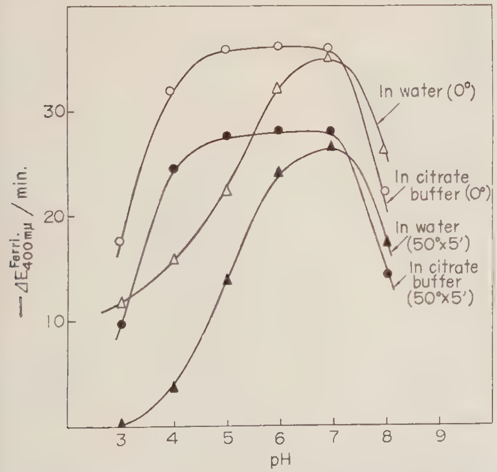


Fig. 6. Effect of pH and heat on stability of alcohol-ferricyanide reducing enzyme.

When inactivated either by lowering or raising the pH or by heat treatment, the color of the enzyme solution changed from red to yellow. Inactivation of the alcohol-ferricyanide reducing enzyme in these ways was in parallel with the decomposition of the cytochrome c-like hemeprotein as judged from the absorption spectrum. Attempts to prepare a sample with the absorption spectra of the hemeprotein but without alcohol-ferricyanide reducing activity were unsuccessful.

Electron Acceptors—Besides ferricyanide, 2, 6-dichlorophenolindophenol, thionine and methylene blue could be more or less reduced by ethanol in the presence of enzyme, indicating that the enzyme could utilize both uni- and di-electron acceptors (Table IV). Neither indigo tetrasulfonate, nile blue nor phenosafranine were reduced.

Table IV

Electron Acceptors of Alcohol-Ferricyanide Reducing Enzyme

Ethanol used as substrate

Electron acceptor	Conc. of enzyme ($E_{280m\mu}$)	Time for decolorization (minutes)
2,6-Dichlorophenolindophenol	6×10^{-5}	6
Potassium ferricyanide	3×10^{-4}	3
Thioninn	3×10^{-3}	15
Methylene blue	3×10^{-3}	420
	3×10^{-2}	8
Indigo tetrasulfonate	3×10^{-2}	>720
Nile blue	3×10^{-2}	>720
Phenosafranine	3×10^{-2}	>720

It was demonstrated manometrically that if a high concentration of enzyme was used, there was oxygen-uptake with ethanol in the presence of methylene blue. In agreement with the result of the previous paper (*2*), in which methylene blue failed to restore the cyanide inhibition of the alcohol oxidation of whole cells and cellular fragments of the micro-organism the oxygen-uptake was optimal at pH 7.0 and was inhibited by cyanide.

Neither TPN nor DPN were reduced by ethanol in the presence of the enzyme, regardless of the purity of the enzyme used. On the contrary, the reduction rate of bovine heart muscle cytochrome c by ethanol was greatly influenced by the purity of the enzyme. Thus eluate from the pH 3-aluminium oxide column had ten times as high ethanol-cytochrome c reducing activity as the preparation at the final step of purification.

Substrate Specificity—The alcohol-ferricyanide reducing enzyme showed a very broad substrate specificity with aliphatic *n*-monoalcohols. Alcohols having five or less carbone atoms in their chain could be rapidly oxidized, but those having longer chains were oxidized more slowly (Table V). Of the unsaturated alcohols tested, allylalcohol was oxidized at a similar rate to saturated alcohols with short chains and propagyl alcohol at a slower rate. However, it should be mentioned that the alcohols which were oxidized at a slow rate were fairly insoluble in water. Therefore, the substrate specificity of the enzyme may well not have been affected by steric hindrance resulting from the length of the straight carbone chains. Iso-form alcohols were not oxidized, indicating that steric hindrance resulted either from the width or height of the alcohol molecules. To study this, aromatic alcohols were tested. Neither phenol nor benzylalcohol were oxidized at all, while phenylethylalcohol and phenylpropylalcohol were fairly well oxidized. The oxidation of the much less water-soluble phenylpropylalcohol and cinnamylalcohol at a faster rate than phenylethylalcohol strongly supports the above suggestion of steric hindrance since the steric hindrance caused by the phenyl radical might be prevented if this radical were located far from the hydroxymethyl radical which becomes attached to the active sites on the enzyme.

Of the dialcohols tested, the enzyme oxidized only 1, 4–butyleneglycol. This was attacked at almost the same rate as ethanol. This suggests that the second hydroxyl radical might show an affinity for the active site of the enzyme and thus inhibit the activity.

TABLE V

Substrate Specificity of Alcohol-Ferricyanide Reducing Enzyme

Substrate	Enzyme	
	$-\Delta E^{\text{Ferri.}}_{400\text{m}\mu}$/min.	Time for decolorization (minutes)
Monoalcohol :		
Saturated :		
Methylalcohol	0.000	>720
Ethylalcohol	0.118	5
n-Propylalcohol	0.076	5
n-Butylalcohol	0.102	5
n-Pentylalcohol	0.104	5
n-Hexylalcohol[1]	—	7
n-Heptylalcohol[1]	—	10
n-Octylalcohol[1]	—	10
n-Nonylalcohol[1]	—	15
n-Decylalcohol[1]	—	15
iso-Propylalcohol	0.000	>720
iso-Butylalcohol	<0.001	>720
iso-Pentylalcohol	<0.003	>720
Unsaturated :		
Allylalcohol	0.101	5
Propagylalcohol[1]	—	32
Dialcohol :		
Ethyleneglycol	<0.001	>720
Propyleneglycol	<0.001	>720
Butyleneglycol (1, 4)	0.105	5
(1, 3)	<0.001	>720
(2, 3)	<0.001	>720
Polyalcohol :		
Glycerol	0.000	—
D-Mannitol	0.000	—
Dulcitol	0.000	—
Aminoalcohol :		
Aminoethylacohol	0.000	—
Thio-alcohol :		
Ethylmercaptane	<0.002	>720
Aromatic alcohol :		
Phenol	0.000	>720
Benzylalcohol	0.000	>720
Phenylethylalcohol	0.005	30
Phenylpropylalcohol[1]	—	22
Cinnamylalcohol[1]	—	11
Monosaccharide :		
L-Arabinose	0.000	—
D-Ribose	0.000	—
D-Xylose	0.000	—
D-Fructose	0.000	—
D-Galactose	0.000	—
D-Glucose	0.000	—
D-Mannose	0.000	—
D-Sorbose	0.000	—
Disaccharide :		
Sucrose	0.000	—
Maltose	0.000	—
Lactose	0.000	—

[1] Added as an emulsion.

The Michaelis constant (Km) was 2.1×10^{-3} moles/liter for ethanol, 2.4×10^{-3} moles/liter for *n*-propanol, 2.3×10^{-3} moles/liter for *n*-butanol, 1.2×10^{-3} moles/liter for *n*-pentanol and 1.3×10^{-3} moles/liter for allylalcohol, in the presence of ferricyanide ($5.8\times10^{-4}M$) (Fig. 7). The Km for ferricyanide was 3.5×10^{-4} moles/liter with ethanol, 2.3×10^{-4} moles/liter with *n*-propanol and 1.6×10^{-4} moles/liter with *n*-butanol, in the presence of $5.8\times10^{-3}M$ solutions of each substrate (Fig. 8).

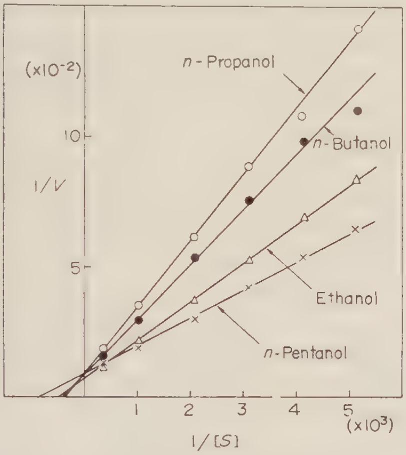


FIG. 7. 1/V-1/[S] curves of alcohol-ferricyanide reducing enzyme with various substrates.

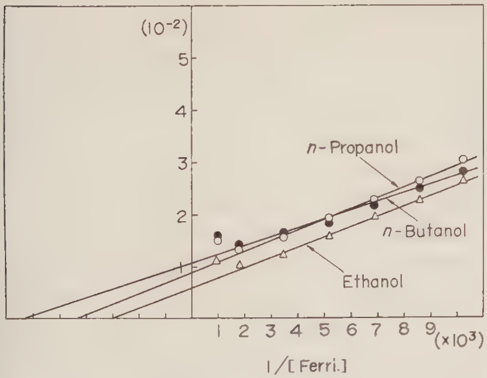


FIG. 8. 1/V-1/[Ferricyanide] curve of alcohol- ferricyanide reducing enzyme with various substrates.

Effect of Ions—The ethanol-ferricyanide reducing activity was lost approximately 40 per cent by 48-hours dialysis against deionized water at 3–4°C. The dialyzed enzyme could be slightly activated by Mg^{++}, while most of the other ions tested were inhibitory (Table VI). NH_4^+, PO_4^{---}, Zn^{++} and Fe^{+++} inhibited the enzyme strongly.

TABLE VI

Effect of Ions on Alcohol-Ferricyanide Reducing Enzyme

Ethanol-ferricyanide reducing activity assayed.

Ion	Inhibition (per cent)	
	Concentration of ion	
	$6\times10^{-4}M$	$3\times10^{-3}M$
NH_4^+	63	92
K^+	0	13
Mg^{++}	0	-5
Ca^{++}	0	0
Zn^{++}	100	100
Fe^{+++}	100	100
PO_4^{---}	47	63
SO_4^{--}	32	37

Effect of Inhibitors—The ethanol-ferricyanide reducing activity was not inhibited by sulfhydryl reagents, indicating that this enzyme may not have a sulfhydryl group in its active area (Table VII). Carbonyl reagents were definitely inhibitory, indicating that a carbonyl radical may be present in the active area. The metal chelating reagents tested were more or less inhibitory, indicating that some metal functions either chemically during the ferricyanide-reducing reaction.

The Mutual Reaction between the Alcohol-Ferricyanide Reducing Enzyme and the Coenzyme-Independent Aldehyde Dehydrogenase—It was shown that neither the coenzyme-dependent nor the coenzyme-independent aldehyde dehydrogenase reduced mammalian cytochrome c, either in the presence or in the absence of coenzymes (TPN and DPN), and that neither enzyme had a hemeprotein-like absorption spectrum (*2–4*). However, a catalytic amount of

TABLE VII

Effect of Inhibitors on Alcohol-Ferricyanide Reducing Enzyme

(Ethanol-ferricyanide reducing activity assayed.

Reagent	Inhibition (per cent)	
	Concentration of reagent	
	$6\times10^{-4}M$	$3\times10^{-3}M$
Sulfhydryl reagents:		
Monoiodoacetate	0	0
p-Chloromercuribenzoate	0	0
Arsenite	0	0
Carbonyl reagents:		
Hydroxylamine	16	26
Semicarbazide	17	36
Hydrogen sulfite	31	40
Metal-chelating reagents:		
Ethylenediamine tetraacetate	5	13
Cyanide	8	23
Azide	7	14
o-Phenanthroline	8	23
Fluoride	32	68
8-Oxy-quinoline	0	7
Pyrophosphate	0	21

the coenzyme-indepent aldehyde dehydrogenase could catalyze the full reduction of the hemeprotein present in the alcohol-ferricyanide reducing enzyme preparation in the presence of acetaldehyde, though the hemeprotein was not reduced by acetaldehyde in the absence of the dehydrogenase. Reduction of the hemeprotein by acetaldehyde was not observed in the presence of a high concentration of the coenzyme-dependent aldehyde dehydrogenase.

DISCUSSION

All of the alcohol-oxidizing enzymes so far purified and characterized have been TPN- or DPN-dependent enzymes. Therefore, because of its coenzyme-independence, the present alcohol-ferricyanide reducing enzyme purified from *Acetobacter* sp. can be a new type of alcohol dehydrogenase. Since in the presence of alcohols this enzyme reduces the naturally occurring cytochrome-553 of the microorganism, it seems suitable to name the enzyme "alcohol-cytochrome-553 reductase (*Acetobacter*)".

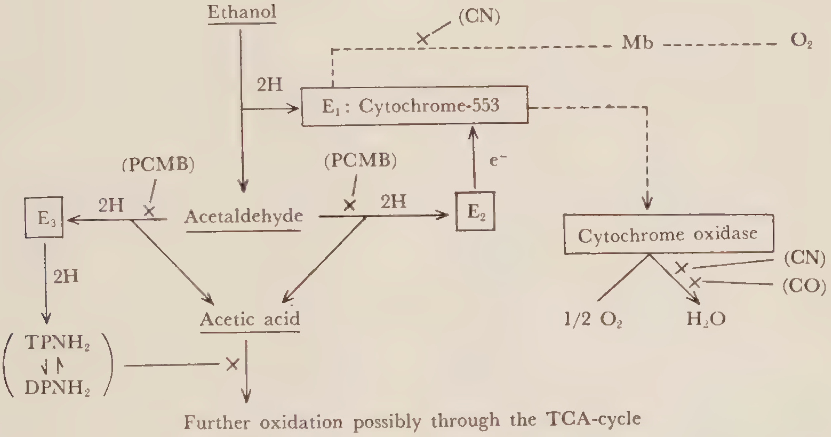
From the constancy of the ratio of the enzyme activity to the hemeprotein content during purification, and the parallel loss of activity and of the cytochrome c-like absorption spectrum of the enzyme preparation, the hemeprotein itself seemes to be responsible for enzyme activity.

An intimate relation between cytochrome and dehydrogenase has been investigated in various dehydrogenases (*10–23*). Of the known dehydrogenases, succinic dehydrogenase of heart muscle and yeast lactic dehydrogenase merit comparison with the alcohol-cytochrome 553 reductase (*Acetobacter*). Succinic dehydrogenase is associated with cytochrome b in heart muscle, but was separated from this by treating with organic solvent and shown to be a flavoprotein. Appleby and Morton have crystallized yeast lactic dehydrogenase and shown that it is a hemeprotein consisting of cytochrome b_2, flavin and nucleotide. On the other hand, Yamashita *et al.* (*20*) have crystallized cytochrome b_2 which has neither enzyme activity nor a flavin, and suggested that yeast lactic dehydrogenase is a complex of cytochrome b_2 and a flavin enzyme. On the basis of these facts, it seems likely that the alcohol-cytochrome 553 reductase obtained here is a complex of dehydrogenase and hemeprotein. However, the proposed structure remain hypothetical and need further experimental confirmation.

Alcohol-cytochrome-553 reductase (*Acetobacter*) can reduce various oxidation-reduction dyes, some being uni- and some di-electron acceptors. This shows that the enzyme has two types of electron outlets. Furthermore, in its crude state, the enzyme can reduce animal cytochrome c much faster than in its purified state. It seemed likely that during purification the enzyme may loose some metal which is an important intermediate in electron transfer from the enzyme to animal cytochrome c. This idea is supported by the role of molybdenum ion in the reduction of cytochrome c by pig liver aldehyde oxidase (*24*, *25*) and by milk xanthine oxidase (*26*). On the other hand, the coenzyme-independent aldehyde dehydrogenase (*Acetobacter*) may have two different kinds of electron-outlets, for like alcohol-cytochrome-553 reductase (*Acetobacter*) (*4*) it can reduce both uni- and di-electron acceptors. According to general ideas on electron transport, in both enzymes the two-electron outlet may be due to some flavine while the one-electron outlet be due to some metal.

From this series of reports, ethanol oxidation by *Acetobacter* sp. may be visualized by the following scheme (Scheme II);

SCHEME II



where E_1, E_2 and E_3 represent alcohol-cytochrome-553 reductase, coenzyme-independent aldehyde dehydrogenase and TPN-dependent aldehyde dehydrogenase. Mb and PCMB are abbreviations for methylene blue and *p*-chloromercuribenzoate respectively. Ethanol is oxidized to acetaldehyde by E_1 and the resulting electrons are successively delivered to the heme iron of the cytochrome-553 which is E_1 itself. The oxidative product, acetaldehyde, is oxidized either by E_2 or by E_3. *Via* the former, the electrons liberated from acetaldehyde are transfered to the heme bound to E_1, cytochrome-553, and *via* the latter they reduce TPN. The microorganism is believed to possess a cytochrome oxidase, since carbon monoxide inhibits the oxidation of ethanol (*2*). Reduced cytochrome-553 is therefore oxidized oxidase and E_1, in agreement with results of the previous study (*2*), in which methylene blue did not restore the ethanol oxidizing activity of either whole cells or a cell-free preparation after treatment with cyanide. It seems likely that *p*-chloromercuribenzoate inhibits the ethanol oxidation of whole cells and a cell-free preparation at E_2 (*2–4*). It is quite possible that the cytochrome system is involved in the ethanol and acetaldehyde oxidation by E_1 and E_2, while the $TPNH_2$ produced by E_3 disturbs further oxidation of acetic acid through the TCA-cycle by upsetting the equilibrium ($TPNH_2 \rightleftharpoons DPNH_2$), thus favoring the back reactions of the coenzyme-dependent dehydrogenases in the TCA-cycle. The acidic pH optima of E_1 and E_2 must also favor acetic acid accumulation by the micro-organism, for, like E_3, in general enzymes involving reaction of coenzymes show pH optima at neutral or higher pHs.

SUMMARY

1. A new type of alcohol-oxidizing enzyme has been purified from *Acetobacter* sp.. The resulting preparation had more than one hundred times as high a specific activity as the cell-free extract. The highly purified enzyme contained a hemeprotein with an absorption spectrum similar to that of cytochrome c, its reduced α-absorption peak being located

at 553 mμ.

2. In the presence of ethanol, the enzyme reduced various oxidation-reduction dyes including ferricyanide and 2, 6-dichlorophenol-indophenol, which were either uni- and di-electron acceptors. TPN and DPN were not reduced at all. The hemeprotein was immediately reduced by the addition of ethanol.

3. Of the buffers tested, the enzyme was most active in citrate buffer and the optimal pH was 3.8. In this buffer, it was fairly stable from pH 5.0 to 7.0 at temperatures below 45°C. Inactivation of the enzyme proceeded in parallel with the break down of the hemeprotein.

4. The enzyme lost 40 per cent of its activity after 24-hours dialysis against deionized water at 3–4°C. Of the ions tested, only Mg^{++} activated the dialyzed enzyme slightly, while NH_4^+, Zn^{++}, Fe^{+++} and PO_4^{---} were strongly inhibitory.

5. The sulfhydryl reagents tested, including *p*-chloromercuribenzoate, did not inhibit the enzyme activity, while all the carbonyl and metal-chelating reagents tested, including semicarbazide and fluoride, were more or less inhibitory.

6. Using ferricyanide as an electron acceptor, the enzyme showed a broad substrate specificity; Almost all the saturated and unsaturated straight chain monoalcohols tested were rapidly oxidized, but iso-alcohols were not. With ferricyanide the Km (moles/liter) was 2.1×10^{-3} for ethanol, 2.4×10^{-3} for *n*-propanol, 2.3×10^{-3} for *n*-butanol, 1.2×10^{-3} for *n*-pentanol and 1.3×10^{-3} for allylalcohol. The Km for ferricyanide was 3.5×10^{-4} with ethanol, 2.3×10^{-4} with *n*-propanol and 1.6×10^{-4} with *n*-butanol.

7. The hemeprotein was rapidly reduced by acetaldehyde in the presence of the coenzyme-independent aldehyde dehydrogenase (*Acetobacter*), but not in its absence.

8. Based upon these results, the steric configuration and the active centers of the enzyme molecule were discussed the likelihood that the hemeprotein itself had enzyme activity indicated. It was proposed to name the enzyme "alcohol-cytochrome-553 reductase (*Acetobacter*)".

9. The electron-transfer system of the micro-organism was discussed in the light of the results of this series of reports and the fact that acetic acid is accumulated to a great extent as an intermediate product of ethanol oxidation was stressed.

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Studies on Protamines

IX. Fractionation of Clupeine

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The heterogeneity of some protamines has been reported since the Kossel's age (*1*). Clear-cut fractionation method of these proteins, however, has not been found before the introduction of countercurrent distribution and alumina chromatography to this field.

Rauen, Stamm and Felix (*2*) were the pioneers, who applied Craig's countercurrent distribution technique to the fractionation of protamines. Using their solvent system, the methyl ester hydrochlorides of clupeine (*2*, *3*) and mugiline β (*4*) were fractionated, but it was not the case of the unesterified protamine sulfates (*2*, *5*). Later, however, using unique solvent systems, the fractionation of clupeine sulfate could be performed independently in our laboratory (*6*) and in the Scanes and Tozer's (*7*).

Meanwhile, the usefulness of alumina chromatography for the fractionation of clupeine was demonstrated by Scanes and Tozer (7). The method was slightly modified by the present authors and was applied to the detection of heterogeneity of iridine (*8*, *9*) and clupeine (*9*) which were obtained from each single fish, respectively.

The present paper deals with the detailed description on the fractionation of clupeine from Pacific herring (*Clupea pallasii*) by the two different methods mentioned above, and also with the characterization of its fractions by the amino acid composition and N-terminal analyses.

EXPERIMENTAL

Material—Clupeine sulfate used for the fractionation was prepared as described before (*6*, *10*).

Alumina Chromatography—The principles of alumina chromatography were described in a previous paper of this series (*8*).

(i) *Analytical chromatography using an unbuffered column*: According to Scanes and Tozer (*7*), 9 g. of Type A alumina (*8*) suspended in distilled water was poured into a chromatographic tube of 0.62 cm. internal diameter, and the column was washed overnight with about ten volumes of distilled water. The height of the column was then adjusted to 31 cm., and 4 to 29 mg. of clupeine samples (sulfate or bicarbonate) dissolved in 1 ml. of distilled water were added to the column. After washing with 1 ml. of distilled water, the column was developed at 25°C with 0.45 *M* aqueous K_2HPO_4 solution at the flow rate of 4 to 5 ml. per hour and every 2.0 ml. fraction was collected.

(ii) *Analytical chromatography using a buffered column*: A 0.62 (or 0.63)×31 cm. column of buffered alumina was made, and 2 to 7 mg. of clupeine samples (sulfate or bicarbonate) were chromatographed according to a previous paper (*8*). 0.45 and 0.48 *M* aqueous K_2HPO_4 were used as eluant buffer for Type A and B alumina, respectively (*8*).

(iii) *Preparative chromatography using an unbuffered column*: Chromatography was done almost similarly as described in part (i), with the exception that 503 mg. of clupeine sulfate was eluted from a 2.54×31 cm. column (Type A alumina) at the flow rate of 50 to 100 ml. per hour and the effluent was collected in 18 ml. fractions (Fig. 1).

(iv) *Preparative Chromatography using a buffered column*: Chromatography was performed almost similarly as described in part (ii), with the exception that 201 mg. of clupeine sulfate was eluted from a 1.88×46 cm. column (Type B alumina) at the flow rate of 42 ml. per hour and the effluent was collected in 14 ml. fractions (Fig. 2).

(v) *Assay of effluent fractions*: For assay of the effluent fractions of clupeine, the retention analysis using ponceau 6RF (*6*), spectrophotometry at 210 mμ

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(8) and the Sakaguchi colorimetry (*11*) were used. In the last case, a weight-color calibration curve was referred to, considering an interference effect on color development by effluents from an alumina column.

Countercurrent Distribution—The principles (*10*) and preliminary experiments (*6*) have already been reported from our laboratory.

(i) *Solvent system*: An aqueous solution containing 0.12 *M* sodium *p*-toluenesulfonate* and 0.40 *M* sodium chloride was equilibrated with an equal volume of *n*-butanol at 22–23°C. Equal volumes from both resulting phases were used as the solvent system.

(ii) *Operation*: All-glass micro distribution equipment (*12*)** with 51 or 52 tubes (1.0 ml. of the lower phase volume) was settled onto an automatic countercurrent distribution machine. The upper phase (1.0 ml.) as mobile phase and the lower phase (2 drops) for co-current were added by hand to the initial tube at every transfer.

A part (19.7 mg.) of clupeine fraction Y sulfate, fractionated through the buffered alumina column (see recovery part), was scattered in the first two tubes, and was distributed until 186 transfers with single withdrawal procedure (*13*) at 22–23°C. A part (2.2 mg.) of clupeine fraction Z II sulfate, fractionated hrough the buffered alumina column (see recovery part), was also distributed until 90 transfers under the ame conditions.

(iii) *Assay of distributed fractions*: In the case of raction Y, each of the distributed fractions remained n the apparatus was dried *in vacuo* over $CaCl_2$-conc. H_2SO_4-NaOH, the residue was dissolved in distilled water, and the solution was analyzed by retention nalysis using ponceau 6RF (*6*); each of the withdrawn raction was analyzed *in situ* by the same way. In the ase of fraction Z II, all fractions distributed were ried *in vacuo* over $CaCl_2$-conc. H_2SO_4-NaOH, and nalyzed by the Sakaguchi colorimetry (*11*).

Recovery of Clupeine from the Chromatographic Effluent–In the case of chromatography using an unbuffered olumn, each of the combined fractions Y and Z Fig. 1) was adjusted to pH 1.8 by HCl to dissolve occulent precipitates usually formed, and was adsorbd on an Amberlite XE-64 (IRC-50, H-form) column. fter desalting with *N* acetic acid, specimens were luted with 0.1 *N* HCl. The eluates were treated with mberlite IRA-400 (H-form) and lyophilized. Clupeine icarbonate thus recovered, was sometimes converted nto sulfate by treating it with sulfuric acid and ethanol.

In the case of chromatography using a buffered column, each of the combined fractions Y, Z I and Z II (Fig. 2) was recovered just in the same manner as described for iridine in a previous paper (*8*). Thus three fractions of clupeine sulfate were obtained.

Recovery of Clupeine from the Distribution System—Each of the combined fractions Y I and Y II (Fig. 6a) was shaken with suitable amounts of ethyl ether and distilled water. The upper phase containing no protein was discarded, and the clupeine transferred into the lower phase was recovered as sulfate through an Amberlite XE-64 column.

N-terminal Analysis—N-terminal residues of clupeine were analyzed by Sanger's DNP-method (*14*).

(i) *Dinitrophenylation*: Samples of clupeine sulfate were dinitrophenylated by the method ("Method 3") described in a previous paper (*15*).

(ii) *Estimation of the molar contents of N-terminal prolyl species*: The ratios ('*R*-values') of optical densities of dinitrophenylated clupeine samples at 390 mμ and 360 mμ were measured in *N* HCl. The molar contents of N-terminal prolyl species in clupeine samples were estimated from the *R*-values according to our previous results (*15*).

(iii) *Identification of N-terminal residues*: Each solution of DNP-clupeine in *N* HCl used for measuring *R*-values was dried *in vacuo* over solid sodium hydroxide in a dark place, and the resultant residue was hydrolyzed with redistilled 6 *N* HCl at 105°C for 16 hours. The hydrolyzate was treated as usual to give ethereal and aqueous phases (*14*). While the aqueous phase contained no DNP-derivative and was discarded, the ethereal phase was chromatographed on a Toyo-Roshi No. 51 filter paper (40×40 cm.) according to Levy (*16*).

Amino Acid Analysis—Amino acid compositions of clupeine fractions were determined by densitometry of paper chromatograms of their acid hydrolyzates in the same manner as described in a previous paper (*8*).

RESULTS AND DISCUSSION

Chromatographic Separation of Clupeine—When clupeine sulfate was eluted from an unbuffered alumina column, essentially the same chromatogram (Fig. 3a), as was described by Scanes and Tozer (*7*), was obtained; two main peaks Y and Z, and a minor peak W appeared.

In the case of rechromatography (Figs. 4a and b) of the sulfate fractions Y and Z recovered from an unbuffered column (Fig. 1), corresponding peaks were formed at such positions as was expected from their original chromatograms, though the separation is not as yet enough. In the case of rechromatography of these fractions in bicarbonate form, however, unexpected patterns, as shown in Figs. 4c and d, were obtained.

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* Recrystallized from hot HCl-ethanol.

** Kindly constructed by Mr. M. Chujo, a lassworker in this university, to whom the authors' hanks are due.

On the other hand, chromatography of clupeine sulfate on a buffered alumina column gave a much better resolution (Figs. 3b and c) as compared with that on an unbuffered one (Fig. 3a). Moreover, on a buffered column, even bicarbonate specimen was satisfactorily rechromatographed (Fig. 5a). A minor peak W, appearing with its hold-up volume from an unbuffered alumina column, disappeared, when clupeine sulfate was eluted from a buffered alumina (Figs. 3b and c). Therefore, it is considered, that this minor fraction is a chromatographic artifact, or a component insoluble in the phosphate buffer used.

By preparative chromatography of clupeine on a buffered column, three fractions of sulfate, namely Y, Z I and Z II, were obtained (Fig. 2). Each of them gave a single

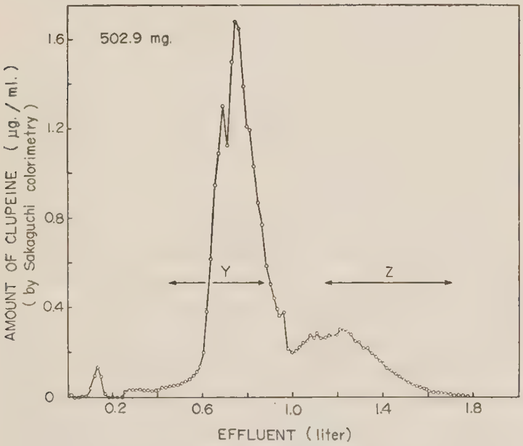

Fig. 1. Preparative chromatography of clupeine sulfate using an unbuffered column. Type A alumina and 0.45 M K_2HPO_4 were used. The recovery was 96%.

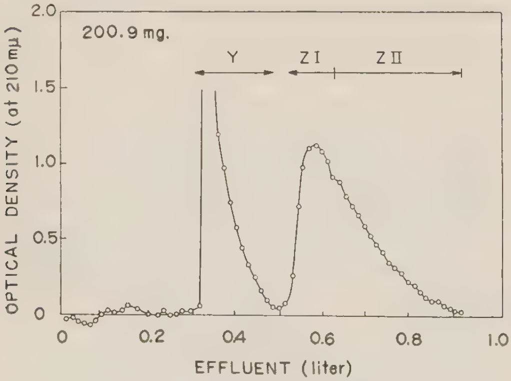

Fig. 2. Preparative chromatography of clupeine sulfate using a buffered column. Type B alumina-0.48 M K_2HPO_4.

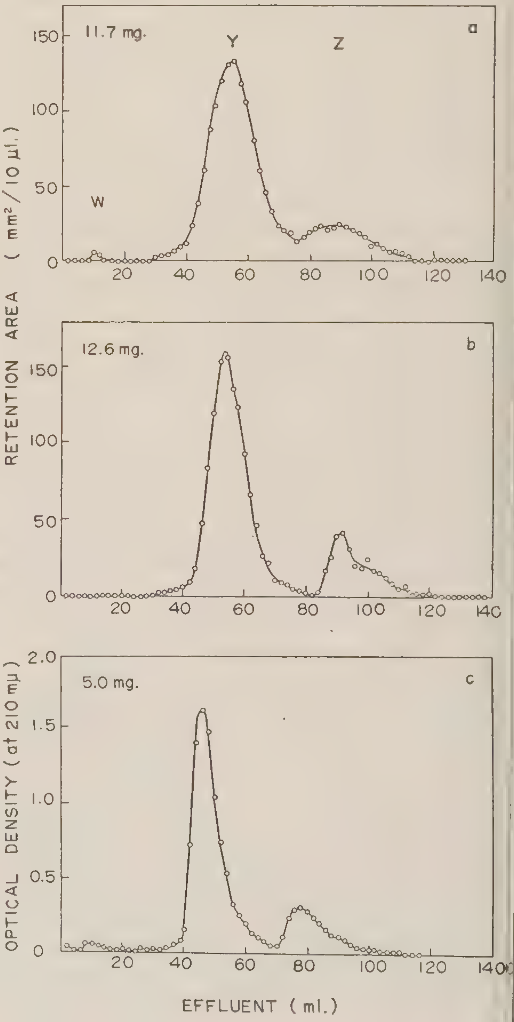

Fig. 3. Analytical chromatography of clupeine sulfate using unbuffered (a) or buffered (b and c) columns. a and b: Type A alumina-0.45 M K_2HPO_4. c: Type B alumina-0.48 M K_2HPO_4.

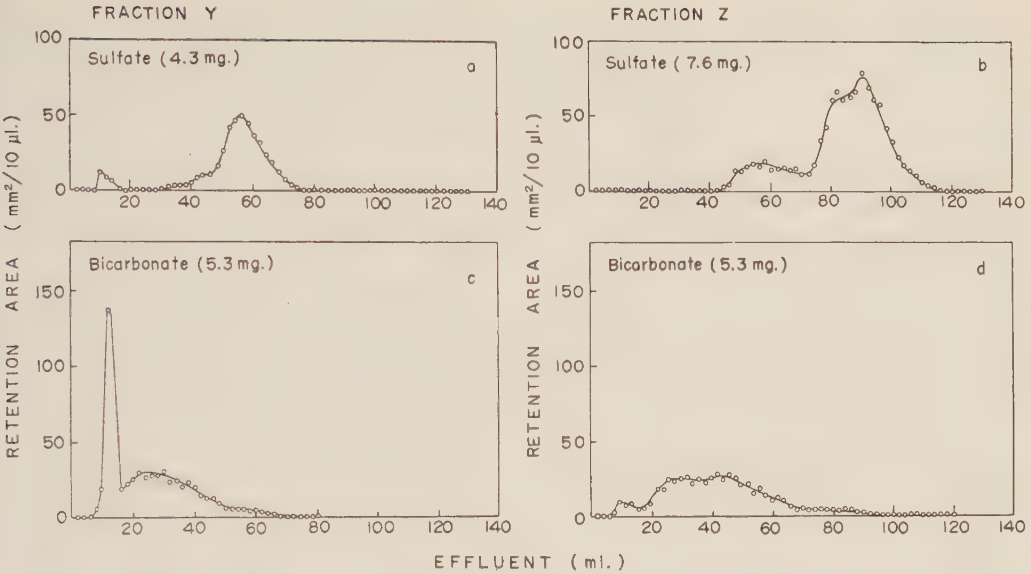

Fig. 4. Analytical rechromatography of clupeine fractions Y (a and c) and Z (b and d) using unbuffered columns. Type A alumina and $0.45 M$ K_2HPO_4 were used. Clupeine sulfate was used in a and b, and the bicarbonate in c and d. Both the salts were prepared by preparative chromatography using an unbuffered column as shown in Fig. 1.

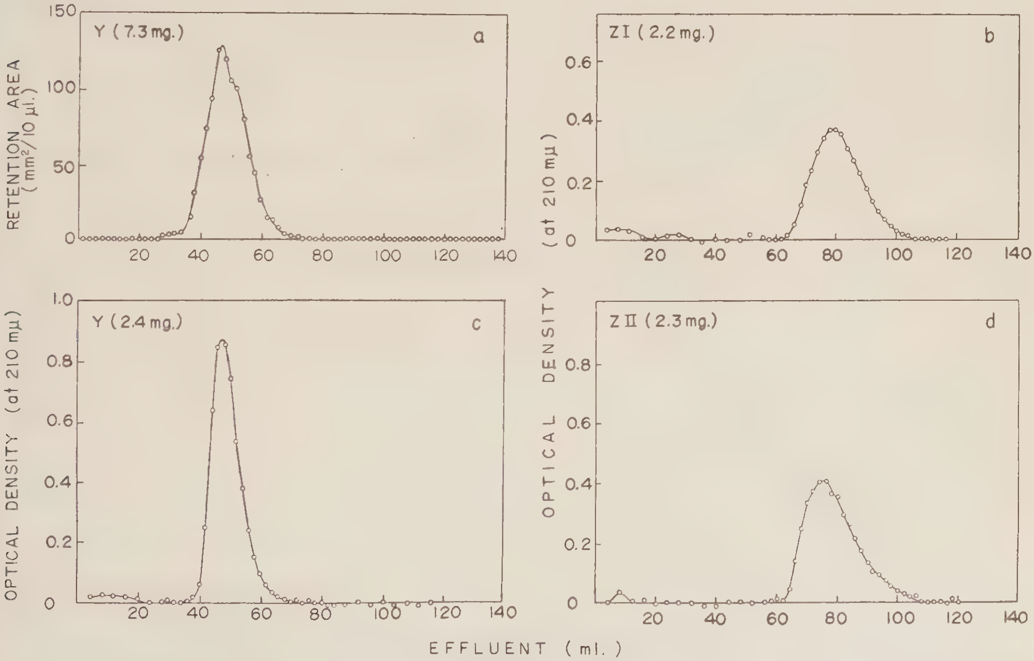

Fig. 5. Analytical rechromatography of clupeine fractions Y (a and c) and Z (b and d) using buffered columns. a: Bicarbonate specimen obtained by preparative chromatography using an unbuffered column (see Fig. 1) was eluted from Type A alumina with $0.45 M$ K_2HPO_4. b, c and d: Sulfate specimens obtained by preparative chromatography using buffered columns (see Fig. 2) were eluted from Type B alumina with $0.48 M$ K_2HPO_4.

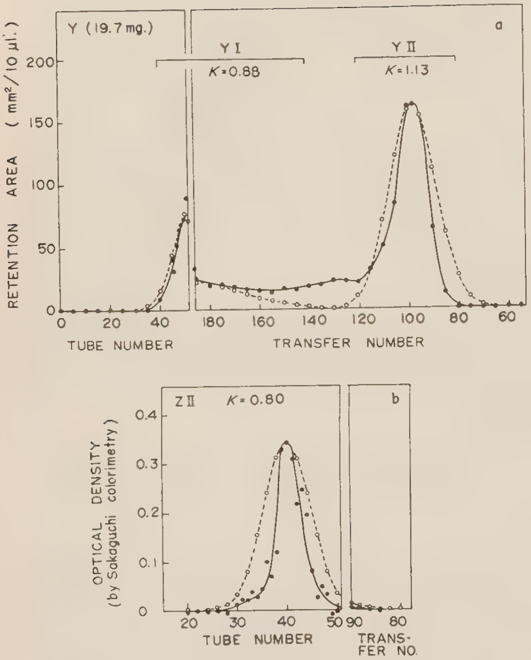

FIG. 6. Countercurrent distribution of clupeine fractions Y (a) and Z II (b) obtained by preparative chromatography using a buffered column (Fig. 2). Solvent system: 0.12 M aqueous sodium p-toluenesulfonate-0.40 M NaCl $vs.$ n-butanol, 22–23°C. —●— experimental curve; --○-- theoretical curve. K: Distribution coefficient.

peak at the expected position after rechromatography on a buffered alumina column (Figs. 5c, b and d). Clupeine thus proved to be composed of some chromatographically different components. These specimens were used for analysis of N-terminal and amino acid composition as well as for further fractionation studies by countercurrent distribution.

Fractionation of Clupeine by Countercurrent Distribution—Clupeine fractions Y and Z separated by alumina chromatography on a buffered column (Fig. 2) gave countercurrent distribution patterns shown in Figs. 6a and b. The Y fraction was thus resolved into two peaks Y I and Y II; these two main fractions, Y I and Y II, were served for N-terminal and amino acid analyses. In contrast, no further fractionation was achieved with Z II fraction as shown in Fig. 6b.

The theoretical patterns (*13*) of Y II and Z II fractions, calculated from the distribution coefficients of 1.13 and 0.80, respectively, were somewhat broader than experimental ones. The reason of this curious phenomenon, that is also observed with serum albumin (*17*) and lactogenic hormone (*18*), remains as yet unknown.

Characterization of Clupeine Fractions—Results

TABLE I

N-Terminal Analysis of Clupeine Fractions[1]

		Unfractionated specimen	Fraction			
			Y I	Y II	Z I	Z II
DNP-Group introduced[2]		1	0.94	0.96	0.90	0.82
DNP-Clupeine	λ max(mμ)	360	357	376	354	354
	R-value	0.75	0.65	0.95	0.54	0.54
N-Terminal	Prolyl (%)	42	24	85	0	0
	Alanyl (%)	58	76	15	100	100

[1] By DNP method. N-Terminal prolyl and alanyl contents were estimated from the R-values (ratios of optical densities at 390/360 mμ) of absorption curves in N HCl of DNP-clupeine specimens (*15*). Identification of the N-terminal residues was performed by two-dimensional paper chromatography of acid hydrolysates of DNP-specimens as described in the text.

[2] Relative amounts (unfractionated specimen=1) calculated from the optical density of DNP-clupeine at 360 mμ in N HCl.

of analyses of N-termini* and amino acid compositions of unfractionated clupeine and its fractions Y I, Y II, Z I and Z II were summarized in Tables I and II.

TABLE II

*Amino Acid Composition of Clupeine Fractions*1)

	Unfractionated specimen	Clupeine fraction			
		Y I	Y II	Z I	Z II
Arginine	(+)	(+)	(+)	(+)	(+)
Proline	(+)	(+)	(+)	(+)	(+)
Alanine	1	1	1	1	1
Serine	0.8_0	1.2_5	0.8_0	0.6_7	0.6_8
Valine	0.8_5	0.3_3	1.0_1	0.8_7	0.8_6
Threonine	0.3_3	0.5_9	0.6_1	0	0
Isoleucine	0.2_1	0.4_8	0.0_6	0	0
Glycine	0.1_3	0.7_9	0.0_4	0	0

1) Measured by densitometry of paper chromatograms of acid hydrolysates colored by ninhydrin and represented as relative values of molar content. (+) means the evident presence of the amino acid, though not measured densitometrically.

It is concluded from these data, that clupeine was fractionated into three fractions, Y I, Y II and Z, whose N-termini and amino acid composition were different from each other; no difference, however, was observed between fractions Z I and Z II. Molecular weight of these fractions seemed almost similar to each other, as estimated from the amounts of DNP-groups introduced into their molecules.

Thus, attention must be paid to the following three main components: Y I, having predominantly N-terminal alanine and seven component amino acids (arginine, proline, alanine, serine, threonine, isoleucine and glycine), Y II, N-terminal proline in the greater part and six component amino acids (arginine, proline, alanine, serine, valine and threonine), and Z, having N-terminal alanine and five kinds of amino acids as components (arginine, proline, alanine, serine and valine). Among the three the component Z has the simplest composition.

TABLE III

*Characterization of Clupeine Fractions Obtained by Different Authors*1)

		Present authors			Scanes and Tozer (*7*)			Felix *et al.* (*3*)			Waldschmidt-Leitz (*19*)
		Y I	Y II	Z	Y-L	Y-H	Z	2nd	3rd	1st	A component
Amino acid composition	Arginine	(+)	(+)	(+)	++++	++++	++++	++++	++++	++++	(+)
	Proline	(+)	(+)	(+)	++	+++	++	++	+++	++	(+)
	Alanine	++	++	++	++	++	++	++	++	+++	(+)
	Serine	++	+	+	++	++	++	++	++	++	(+)
	Valine	±	++	++	±	++	+	−	++	++	(+)
	Threonine	+	+	−	+	+	−	++	+	−	−
	Isoleucine	+	±	−	+	±	−	+	−	−	−
	Glycine	+	±	−	+	±	−	+	−	−	−
N-Terminal residues		(Pro) Ala	Pro (Ala)	Ala	Pro Ala		Ala	Pro	Ala, Ser, Thr	Pro	Pro

1) + and ++ *etc.* represent relative values of amino acid content, while (+) means the presence of the amino acid. (Pro) or (Ala) represents its existence in much lesser amount.

Characteristics of clupeine fractions obtained by different authors are summarized in Table III. In spite of a remarkable analogy in amino acid composition of each corresponding fraction obtained by different authors, interesting differences are observed in the N-terminal residues. For example, Z fractions of the present authors and Scanes and Tozer (*7*), as well as Felix's first component (*3*) and a component of Waldschmidt-Leitz (*19*), are all characterized by the absence of threonine, isoleucine and glycine in their amino acid compositions, while the N-terminal residue of the two formers is alanine and that of the two latters is proline. This distinct difference would be a reflection of the differences in biological species of fish from which clupeine specimens were obtained. The species of fish used in our study is Pacific herring, *Clupea pallasii Cuviet et Valencienne*, whereas that used in Felix's and Waldschmidt-Leitz's laboratories is Atlantic herring, *Clupea harengus Linnaeus.* Although the species of fish used by Scanes and Tozer is not described, it may belong to *Clupea pallasii*, judging from the characteristics of their clupeine.

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* N-terminal alanine was identified as DNP-alanine, and N-terminal proline as 2, 4-dinitrophenol, after acid hydrolysis of DNP-clupeine specimens.

Table IV

*Possible Peptide Composition in Each Clupeine Component*1)

	Peptide	Clupeine component		
		Y I	Y II	Z
N-Terminal sequences	Pro-Arg-Arg	−	+	−
	Ala-Arg-Arg	+	−	+
Dipeptide sequence consisting of monoamino-monocarboxylic acids	Pro-Ileu	+	−	−
	Pro-Val	−	+	+
	Val-Ser	−	+	+
	Ala-Ser	+	+	+
	Ala-Gly	+	−	−
	Ser-Ser	+	+	+
	Thr-Thr	+	+	−

1) + and − show the possibility and no possibility of presence of the peptide sequence, respectively, in the clupeine component.

Moreover, it has recently been reported from our laboratory (*20*), that several dipeptide sequences consisting of monoamino-monocarboxylic acids were found in the tryptic hydrolysate of unfractionated clupeine, *i. e.* prolylisoleucine, prolylvaline, valylserine, alanylserine, alanylglycine, serylserine and threonylthreonine. Considering the results together with those of the N-terminal sequences of unfractionated clupeine, alanylarginylarginine and prolylarginylarginine (*21*, *22*), the three clupeine components, Y I, Y II and Z, may possibly contain the sequences as illustrated in Table IV. It is clear from this table, that the component Z must have a very simple peptide composition. Thus studies on amino acid sequences of the Z component are now in progress in our laboratory.

SUMMARY

1. Clupeine prepared from Pacific herring (*Clupea pallasii*) was chromatographed on buffered alumina with an eluant of $0.45\,M$ aqueous dipotassium hydrogen phosphate, and was fractionated to two main fractions, Y and Z.

2. The fraction Y was further separated to two fractions, Y I and Y II, by countercurrent distribution technique using a solvent system consisting of *n*-butanol, aqueous sodium *p*-toluenesulfonate and sodium chloride. The fraction Z could not further be fractionated either by chromatography or by countercurrent distribution.

3. Analyses of N-terminal residues and amino acid compositions of the fractions Y I, Y II and Z were performed. Thus these fractions proved to be different from one another in their N-termini and amino acid compositions.

4. All the fractions contained arginine, proline, alanine and serine. The fraction Y I contained additional amino acids, threonine, isoleucine, glycine and a small amount of valine. The fraction Y II additionally contained valine and threonine as well as traces of isoleucine and glycine. Fraction Z contained only valine in addition. The main N-terminus of the fraction Y I was alanine, and that of the fraction Y II was proline. The fraction Z contained only alanine in its N-terminus.

5. The present results on the characteristics of clupeine fractions were compared with

those obtained in some other laboratories, and some discussions were made on the species specificity of clupeine.

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Preparation of Reduced, Activator-Free Papain by Para-Thiocresol Treatment

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Papain is one of typical SH enzymes, and its maximal proteolytic activity is obtained only in the presence of reducing agents, such as cysteine, glutathione, or cyanide. In the study on the sulfhydryl properties of papain, however, it is often required to remove these reducing agents from the enzyme solution. For removal of the activator, the following methods have been employed, that is, fractional precipitation with organic solvent (*1*), dialysis under an anaerobic condition (*2*), or treatment with the aid of PCMB-saturated ion-exchange resin (*3*). Recently, an alternative method of producing reduced, ion-free papain was afforded by ion-exchange technique (*4*). Nevertheless, all of these techniques are rather complicated. In the present paper a simpler new method is proposed for preparation of reduced papain.

Purified papain was prepared from a commercial papaya latex by a slightly modified method of Kimmel and Smith (*5*). Oxidized papain was obtained by treating 0.5% papain solution with 0.001 M I_2 in 0.1 M KI solution for half an hour at 0°C, and then dialyzed against deionized water for 5 days.

Para-thiocresol solution was prepared by dissolving 56 mg. of *p*-thiocresol (PTC) into 10 ml. of distilled toluene under a current of nitrogen.

Measurement of the proteolytic activity was carried out by the method of Stockell (*6*) and the modified method of Kunitz *et al.* (*7*). The content of papain was determined from its absorption at 278 mμ. The thiol groups were estimated by the methods of Boyer (*1*).

Fifty mg. of the oxidized papain was dissolved into 10 ml. of 0.05 M citrate buffer, pH 5.0, in a Thunberg tube. After the substitution of air with nitrogen, the papain solution was mixed with the same volume of the PTC solution, which had been placed in sidearm cap, and oscillated at 100 strokes per minute for about 5 minutes. Then, the clear upper layer was decanted off and the emulsified lower one was placed on a filter paper (Toyoroshi No. 131) which was previously saturated with water. If the filtrate was not transparent

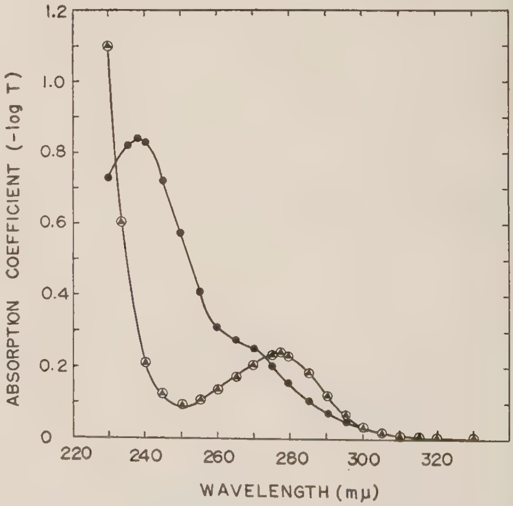

Fig. 1. Absorption curves of PTC, oxidized papain and PTC-reduced papain at pH 7.0.
—●—: 8.6×10^{-5} M PTC in 50 per cent methanol (volume/volume). —▲—: 1.1×10^{-5} M oxydized papain in 10^{-3} M phosphate buffer. —○—: 1.1×10^{-5} M PTC-reduced papain in 10^{-3} M phosphate buffer.

TABLE I

Proteolytic Activity of Papain and Number of Reactive Thiol Groups as Estimated Spectrophotometrically

	Papain		
	Oxidized	Reduced with PTC	Reduced with cysteine[2]
Proteolytic Activity (per cent)[1]	2.2 ±1.5	97.1 ±2.1	100
Thiol content	0.06±0.02	0.93±0.02	—

1) Figures illustrate the activity of papain as per centage of full activity.

2) Papain was proactivated with $5 \times 10^{-2} M$ cysteine (pH 5.0) for half an hour.

enough, filtration had to be repeated after addition of a small amount of acid clay to the solution.

As illustrated in Fig. 1, there was no trace of PTC in the filtrate. During this procedure approximately a half cystine per mole of papain was reduced, and the maximal proteolytic activity was gained as shown in Table I. The results consists with that of Finkle *et al.* (*8*).

In the course of the reduction procedure, the following cares must be taken to avoid the denaturation of papain, *i.e.*, to keep pH within the limits of 3 to 9, ionic strength at 0.15 or less, temperature at lower than 10°C, and frequency in the shaking less than 100 strokes per minute.

Preservation of the enzyme solution under anaerobic conditions leaves the enzyme fully active for several days.

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Effect of Growth Substrates on Levels of Glyoxylate By-pass Enzymes in a Glutamate Forming Bacterium, *Brevibacterium flavum* No. 2247

It has been demonstrated by many workers (*1–7*) that the levels of isocitratase in bacterial extracts vary widely with the substrate on which the cell are grown. For example, relative specific activities of isocitratase were 1.00, <0.003, 0.047 and 0.032 for the extracts of *Pseudomonas ovalis* Chester grown on acetate, glucose, citrate and alanine, respectively (*3*). From these results, it has been suggested that the enzyme is adaptive and operates only when net formation of C_4-compounds from acetate is necessary for growth. The present authors have previously demonstrated isocitratase activity in the glucose-grown cells of *Brevivacterium flavum* No 2247, which accumulates a considerable amount of glutamate from acetate as well as from glucose in the presence of ammonium salts (*8–9*). Therefore, it would be valuable to make comparison of the enzyme activity between the extracts of cell grown on acetate and those obtained from cell grown on glucose.

Br. flavum No. 2247 grown in the previously described media (*8*, *10*) was harvested by centrifugation, washed with 0.2% KCl, and lyophilized. Cell-free extract was prepared from the lyophilized cells by the method described previously (*11*). Protein concentration was estimated by the spectrophotometric method of Warburg and Christian (*12*). Isocitratase was measured by a modification of the method of Smith add Gunsalus (*2*, *8*), and malate synthetase by the method of Dixon and Kornberg (*13*). The specific activities of isocitratase and of malate synthetase thus obtained were: 8.8, 1.3 μmoles/mg. protein/hour for the glucose-grown cells; 48, 3.1 μmoles/mg. protein/hour for the acetate-grown cells, respectively. These results might suggest that isocitratase as well as malate synthetase of *Br. flavum* is constitutive, although many studies have shown isocitratase to be adaptively formed (*1–7*).

On the other hand, it has also been proposed that the formation and the activity of isocitratase can be controlled by feed-back mechanisms maintaining the dicarboxylic acids at a level that may assure continuous functioning of the TCA cycle (*5*, *14*). In the cells grown on glucose, the dicarboxylic acids of the TCA cycle, some of which are known to repress the formation of isocitratase, would be supplied continuously by carbon dioxide fixation to pyruvate and/or phosphopyruvate derived from glucose, whereas this is not the case with the cells grown on acetate. Consequently, the formation of isocitratase would be repressed in the cells grown on glucose, while not repressed in the cells grown on acetate. Similarly, the lack of α-ketoglutaric dehydrogenase system, as is the case with *Br. flavum*, would result in lowering the intracellular level of the compounds such as succinate and thus stimulate the formation of isocitratase even if glucose is the carbon source. Therefore, the present result seems to be in better agreement with the feed-back mechanisms mentioned above, than with the suggestion that isocitratase is constitutive in the one case, while adaptive in the other, under the genetic control.

In either case, the glyoxylate by-pass in the glucose-grown *Br. flavum* as well as carbon dioxide fixation to C_3-compounds seems to serve a function in the supply of C_4-compounds required for the glutamate formation (*11*).

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Presence of Formyl-residue as the Form of N-Formyl-Hexosamine in the Urinary Glycoprotein

We have recently isolated an abnormal urinary glycoprotein (I) situated at γ-position on electrophoresis which was obtained from the most carbohydrate rich, but sialic acid poor fraction (Fr. 4) of the urinary glycoprotein adsorbable on benzoic acid (*1*). In the course of study on the quantitative precipitation between "I" and the γ_2-globlin of anti-"I"-rabbit sera, we have found the precipitation (the system: 45 μg. of "I", 300 μg. of γ_2-globulin of anti-"I" per 0.12 ml. of saline) was inhibited by addition of 200 μg. of glucosamine hydrochloride or galactosamine hydrochloride but not even by 2000 μg. of N-acetyl-derivatives of those amino-sugars or by the same amount of galactose (*2*), since then efforts has been paid to elucidate the kinds of acyl-residue or the mode of existence of amino nitrogene of hexosamines in the glycoprotein and have encountered with a finding that the formyl residue is certainly present possibly as the N-formyl-hexosamine basing on the following facts;

1. N-formyl-D-glucosamine synthesized according to Kuhn (*3*) showed much stronger potency in inhibition of the precipitation between "I" and γ_2-globlin of anti-"I"-rabbit sera (500 μg.) than that of N-acetyl-derivatives of hexosamines (2000 μg.).

2. Hydrolysis of "I" with 25% *p*-toluenesulfonic acid (100°C., 3 hours.), was followed by repeated distillation into alkaline water under 5.5 mmHg. at 100°C (*4*). After being treated the distillate to convert the acids into hydroxamates through methyl ester (*5*) examination on paper chromatography in three different solvent was identified two spots: a distinctive one exactly corresponding to the formyl-hydroxamate and the other faint spot corresponding to the acetyl-hydroxamate. The destillate gave reducing properties in hot ammoniacal silver nitrate, mercuric chloride and positive chromotropic acid reaction after being reduced with hydrochloric acid and zinc dust (Feigl's test) (*6*).

3. Neither serine nor threonine but aspartic acid and alanine were found as the N-terminal amino acids of "I" (by DNP method), nevertheless "I" gave considerable amount of ammonia on periodate oxidation during 24 hours (89 per cent of total hexosamine N) no ammonia was evolved by mere incubation of "I" with alkaline water. It has been confirmed that authentic N-formyl-D-glucosamine evolve ammonia as much as 80.1% of the total N on periodate oxidation during 24 hrs., this is the same order of the value of D-glucosamine hydrochloride (82.5 per cent of total N) but is much greater than N-acetyl-D-glucosamine (37.5 per cent of total N) when determined according to Aminoff and Morgan (*7*).

Taken altogether it is considered that hexosamine must be present as N-formyl-hexosamine but not as usual N-acetyl-form. The same finding was observed on the normal γ-glycoprotein corresponding to this abnormal glycoprotein, it is considered, however, on preliminary quantitative observation that the immunological and other biological specificity is largely governed by the raito of the amount of acetyl- and N-formyl-residue in those families of glycoproteins.

Thus it appears to be the first recognition of a new type of acyl-residue in muco-substances of higher vertebrae although Miles and Pirie (*8*) reported in 1939 a presence of N-formyl residue in the polysaccharide of *Brucella melitensis* merely basing on the appearance of amino groups and free formic acid on mild acid hydrolysis.

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